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| (51) International Patent Classification ⁶: C12N 15/53, 9/06, C12Q 1/68, A61K 48/00 | A1 | (11) International Publication Number: WO 95/33054 (43) International Publication Date: 7 December 1995 (07.12.95) |
| (21) International Application Number: PCT/CA95/00314 (22) International Filing Date: 25 May 1995 (25.05.95) (30) Priority Data: 9410620.0 26 May 1994 (26.05.94) GB (71) Applicant (for all designated States except US): MCGILL UNIVERSITY [CA/CA]; 845 Sherbrooke Street West, Montréal, Québec H3A 1B1 (CA). (72) Inventors; and (75) Inventors/Applicants (for US only): ROZEN, Rima [CA/CA]; 9 Fairfield Crescent, Montréal West, Québec H4X 1R5 (CA). GOYETTE, Philippe [CA/CA]; 4368 Harvard, Montréal, Québec H4A 2X1 (CA). (74) Agent: COTE, France; Swabey Ogilvy Renault, Suite 1600, 1981 McGill College Avenue, Montréal, Québec H3A 2Y3 (CA). | | (81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TT, UA, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ, UG). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i> |
| (54) Title: cDNA FOR HUMAN METHYLENETETRAHYDROFOLATE REDUCTASE (57) Abstract The present invention relates to a cDNA probe for human methylenetetrahydrofolate reductase (MTHFR), and its uses. The probe of the present invention may be used for the identification of sequence abnormalities in patients with severe or mild MTHFR deficiency, including cardiovascular patients and patients with neurologic symptoms. A human MTHFR protein which hybridizes to the probe of the present invention may be used for therapy of MTHFR-deficiency patients by biochemical or pharmacological approaches. | | |

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- 1 -

CDNA FOR HUMAN METHYLENETETRAHYDROFOLATE REDUCTASE**BACKGROUND OF THE INVENTION****(a) Field of the Invention**

5 The invention relates to a cDNA probe for human methylenetetrahydrofolate reductase (MTHFR), and its uses.

(b) Description of Prior Art

10 Folic acid derivatives are coenzymes for several critical single-carbon transfer reactions, including reactions in the biosynthesis of purines, thymidylate and methionine. Methylenetetrahydrofolate reductase (MTHFR; EC 1.5.1.20) catalyzes the NADPH-linked reduction of 5,10-methylenetetrahydrofolate to
15 5-methyltetrahydrofolate, a co-substrate for methylation of homocysteine to methionine. The porcine liver enzyme, a flavoprotein, has been purified to homogeneity; it is a homodimer of 77-kDa subunits. Partial proteolysis of the porcine peptide has revealed two
20 spatially distinct domains: an N-terminal domain of 40 kDa and a C-terminal domain of 37 kDa. The latter domain contains the binding site for the allosteric regulator S-adenosylmethionine.

 Hereditary deficiency of MTHFR, an autosomal
25 recessive disorder, is the most common inborn error of folic acid metabolism. A block in the production of methyltetrahydrofolate leads to elevated homocysteine with low to normal levels of methionine. Patients with severe deficiencies of MTHFR (0 -20% activity in
30 fibroblasts) can have variable phenotypes. Developmental delay, mental retardation, motor and gait abnormalities, peripheral neuropathy, seizures and psychiatric disturbances have been reported in this group, although at least one patient with severe MTHFR deficiency was asymptomatic. Pathologic changes in the
35 severe form include the vascular changes that have

- 2 -

been found in other conditions with elevated homocysteine, as well as reduced neurotransmitter and methionine levels in the CNS. A milder deficiency of MTHFR (35-50% activity) has been described in patients
5 with coronary artery disease (see below). Genetic heterogeneity is likely, considering the diverse clinical features, the variable levels of enzyme activity, and the differential heat inactivation profiles of the reductase in patients' cells.

10 Coronary artery disease (CAD) accounts for 25% of deaths of Canadians. Cardiovascular risk factors (male sex, family history, smoking, hypertension, dyslipoproteinemia and diabetes) account for approximately 60 to 70% of our ability to discriminate CAD
15 patients from healthy subjects. Elevated plasma homocysteine has also been shown to be an independent risk factor for cardiovascular disease.

Homocysteine is a sulfhydryl-containing amino acid that is formed by the demethylation of
20 methionine. It is normally metabolized to cysteine (transsulfuration) or re-methylated to methionine. Inborn errors of metabolism (as in severe MTHFR deficiency) causing extreme elevations of homocysteine in plasma, with homocystinuria, are associated with pre-
25 mature vascular disease and widespread arterial and venous thrombotic phenomena. Milder elevations of plasma homocysteine (as in mild MTHFR deficiency) have been associated with the development of peripheral vascular disease, cerebrovascular disease and
30 premature CAD.

Homocysteine remethylation to methionine requires the folic acid intermediate, 5-methyltetrahydrofolate, which is produced from 5,10-methylenetetrahydrofolate folate through the action of 5,10-
35 methylenetetrahydrofolate reductase (MTHFR). Defi-

ciency of MTHFR results in an inability to metabolize homocysteine to methionine; elevated plasma homocysteine and decreased methionine are the metabolic consequences of the block. Severe deficiencies of MTHFR (less than 20% of activity of controls) as described above, are associated with early-onset neurologic symptoms (mental retardation, peripheral neuropathy, seizures, etc.) and with atherosclerotic changes and thromboembolism. Milder deficiencies of MTHFR (35-50% of activity of controls), with a thermolabile form of the enzyme, are seen in patients with cardiovascular disease without obvious neurologic abnormalities.

In a survey of 212 patients with proven coronary artery disease, the thermolabile form of MTHFR was found in 17% of the CAD group and 5% of controls. In a subsequent report on 339 subjects who underwent coronary angiography, a correlation was found between thermolabile MTHFR and the degree of coronary artery stenosis. Again, traditional risk factors (age, sex, smoking, hypertension, etc.) were not significantly associated with thermolabile MTHFR. All the studies on MTHFR were performed by enzymatic assays of MTHFR in lymphocytes, with measurements of activity before and after heat treatment to determine thermolability of the enzyme.

Since 5-methyltetrahydrofolate, the product of the MTHFR reaction, is the primary form of circulatory folate, a deficiency in MTHFR might lead to other types of disorders. For example, periconceptual folate administration to women reduces the occurrence and recurrence of neural tube defects in their offspring. Neural tube defects are a group of developmental malformations (meningomyelocele, anencephaly, encephalocele) that arise due to failure of closure of the neural tube. Elevated levels of plasma homocysteine have

- 4 -

been reported in mothers of children with neural tube defects. The elevated plasma homocysteine could be due to a deficiency of MTHFR, as described above for cardiovascular disease.

5 Neuroblastomas are tumors derived from neural crest cells. Many of these tumors have been reported to have deletions of human chromosome region 1p36, the region of the genome to which MTHFR has been mapped. It is possible that MTHFR deletions/mutations are
10 responsible for or contribute to the formation of this type of tumor. MTHFR abnormalities may also contribute to the formation of other types of tumors, such as colorectal tumors, since high dietary folate has been shown to be inversely associated with risk of
15 colorectal adenomas.

 MTHFR activity is required for homocysteine methylation to methionine. Methionine is necessary for the formation of S-adenosylmethionine, the primary methyl donor for methylation of DNA, proteins, lipids,
20 neurotransmitters, etc. Abnormalities in MTHFR might lead to lower levels of methionine and S-adenosylmethionine, as well as to elevated homocysteine. Disruption of methylation processes could result in a wide variety of conditions, such as neoplasias, developmental
25 anomalies, neurologic disorders, etc.

 Although the MTHFR gene in *Escherichia coli* (*metF*) has been isolated and sequenced, molecular studies of the enzyme in higher organisms have been limited without the availability of a eukaryotic cDNA.
30 In this communication, we report the isolation of a human cDNA for MTHFR, its chromosomal assignment, and the identification of mutations in MTHFR-deficient patients. This report represents the first molecular description of mutations in MTHFR deficiency.

- 5 -

It would be highly desirable to be provided with a cDNA probe for human methylenetetrahydrofolate reductase (MTHFR). This probe would be used for identification of sequence abnormalities in individuals with severe or mild MTHFR deficiency, including cardiovascular patients and patients with neurologic symptoms or tumors. The probe would also be used in gene therapy, isolation of the gene, and expression studies to produce the MTHFR protein. The probe would also provide the amino acid sequence of the human MTHFR protein, which would be useful for therapy of MTHFR deficiency by biochemical or pharmacological approaches.

It would be highly desirable to be provided with a molecular description of mutations in methylenetetrahydrofolate reductase deficiency.

SUMMARY OF THE INVENTION

One aim of the present invention is to provide a cDNA probe for human methylenetetrahydrofolate reductase (MTHFR).

Another aim of the present invention is to provide a molecular description of mutations in methylenetetrahydrofolate reductase deficiency.

Another aim of the present invention is to provide a nucleic acid and amino acid sequence for human methylenetetrahydrofolate reductase.

Another aim of the present invention is to provide potential therapy for individuals with methylenetetrahydrofolate reductase deficiency.

Another aim of the present invention is to provide a system for synthesis of MTHFR protein in vitro.

A further aim of the present invention is to provide for a technology/protocol for identification of sequence changes in the MTHFR gene.

BRIEF DESCRIPTION OF THE DRAWINGS

5 Figs. 1A to 1E illustrate the first cDNA coding sequence for methylenetetrahydrofolate reductase (MTHFR);

10 Fig. 2 is the alignment of amino acids for human methylenetetrahydrofolate reductase (MTHFR), the *metF* genes from *E. coli* (ECOMETF), and *S. typhimurium* (STYMETF), and an unidentified open reading frame in *Saccharomyces cerevisiae* that is divergently transcribed from an excision repair gene (*ysRAD1*);

15 Figs. 3A and 3B illustrate the sequencing and restriction enzyme analysis for the Arg to Ter substitution;

20 Figs. 4A and 4B illustrate the sequencing and restriction enzyme analysis for the Arg to Gln substitution;

25 Figs. 5A and 5B illustrates the sequence change and restriction enzyme analysis for the alanine to valine substitution;

30 Fig. 6 illustrates the total available sequence of human MTHFR cDNA;

Figs. 7A and 7B illustrate the expression analysis of MTHFR cDNA in *E. coli*, respectively (7A) the Western blot of bacterial extracts and tissues, and (7B) the thermolability assay of bacterial extracts;

Figs. 8A to 8D illustrate the identification of a 5' splice site mutation leading to a 57 bp in-frame deletion of the cDNA;

Figs. 9A to 9D illustrate the diagnostic restriction endonuclease analysis of 4 mutations;

Figs. 10A to 10D illustrate the ASO hybridization analysis of 2 mutations; and

- 7 -

Fig. 11 illustrates the region of homology between human methylenetetrahydrofolate reductase (MTHFR) and human dihydrofolate reductase (DHFR).

5 DETAILED DESCRIPTION OF THE INVENTION

Sequencing of peptides from porcine MTHFR

Homogeneous native porcine MTHFR was digested with trypsin to generate a 40 kDa N-terminal fragment and a 31 kDa C-terminal fragment; the 31 kDa fragment is a proteolytic product of the 37 kDa fragment. The fragments were separated by SDS-PAGE, electroeluted, and the denatured fragments were digested with lysyl endopeptidase (LysC). The resulting peptides were separated by reversed-phase HPLC and subjected to sequence analysis by Edman degradation (details contained in Goyette P et al., *Nature Genetics*, 1994, 7:195-200).

20 **Isolation and sequencing of cDNAs**

Two degenerate oligonucleotides were synthesized based on the sequence of a 30 amino acid porcine MTHFR peptide (first underlined peptide in Fig. 2). These were used to generate a 90 bp PCR product, encoding the predicted peptide, from reverse transcription-PCR reactions of 500 ng pig liver polyA+ RNA. A pig-specific (non-degenerate, antisense) PCR primer was then synthesized from this short cDNA sequence. Using this primer and a primer for phage arms, a human liver λ gt10 cDNA library (Clontech) was screened by PCR; this technique involved the generation of phage lysate stocks (50,000 pfu) which were boiled for 5 mins and then used directly in PCR reactions with these two primers. PCR fragments were then sequenced directly (Cycle Sequencing™ kit, GIBCO), and a positive clone was identified by com-

parison of the deduced amino acid sequence to the sequence of the pig peptide (allowing for inter-species variations). The positive stock was then replated at lower density and screened with the radio-labelled positive PCR product by plaque hybridization until a well-isolated plaque was identified. Phage DNA was purified and the insert was then subcloned into pBS+ (Bluescript) and sequenced on both strands (Cycle Sequencing™ kit, GIBCO and Sequenase™, Pharmacia). The deduced amino acid sequence of the human cDNA was aligned to the porcine peptide sequences, the metF genes from *E.coli* (*ecometf*, accession number V01502) and *S. typhimurium* (*stymetf*, accession number X07689) and with a previously unidentified open reading frame in *Saccharomyces cerevisiae* that is divergently transcribed with respect to the excision repair gene, *ysRAD1* (accession number K02070). The initial alignments were performed using BestFit™ in the GCG computer package, and these alignments were adjusted manually to maximize homologies.

In summary, degenerate oligonucleotide primers were designed to amplify a sequence corresponding to a 30-amino acid segment of a porcine peptide from the N-terminal region of the enzyme (first porcine peptide in Fig. 2). A 90-bp porcine cDNA fragment was obtained from reverse transcription/PCR of pig liver RNA. Sequencing of the PCR fragment confirmed its identity by comparison of the deduced amino acid sequence to the porcine peptide sequence. A nondegenerate oligonucleotide primer, based on the internal sequence of the porcine cDNA, was used in conjunction with primers for the phage arms to screen a human liver λ gt10 cDNA library by PCR. The insert of the positive clone was isolated and sequenced. The

sequence consisted of 1266 bp with one continuous open reading frame.

Homology with MTHFR in other species

5 The deduced amino acid sequence of the human cDNA was aligned with the *metF* genes from *E.coli* and *S.typhimurium*, as well as with a previously unidentified ORF in *Saccharomyces cerevisiae* that is divergently transcribed with respect to the excision repair
10 gene, *ysRAD1* (Fig. 2). The sequences homologous to 5 porcine peptides are underlined in Fig. 2. Three segments (residues 61-94, 219-240, and 337-351) correspond to internal peptide sequence from the N-terminal 40 kDa domain of the porcine liver enzyme. Residues
15 374-393 correspond to the upstream portion of the LysC peptide from the C-terminal domain of the porcine liver enzyme that is labeled when the enzyme is irradiated with UV light in the presence of (³H-methyl)AdoMet; as predicted from the AdoMet labeling
20 studies, this peptide lies at one end (N-terminal) of the 37 kDa domain. A fifth region of homology (residues 359-372) was also identified, but the localization of the porcine peptide within the native protein had not been previously determined.

25 Methylenetetrahydrofolate reductase (MTHFR) is an enzyme involved in amino acid metabolism, that is critical for maintaining an adequate methionine pool, as well as for ensuring that the homocysteine concentration does not reach toxic levels. The high degree
30 of sequence conservation, from *E.coli* to *Homo sapiens*, attests to the significance of MTHFR in these species. The enzyme in *E.coli* (encoded by the *metF* locus) is a 33 kDa peptide that binds reduced FAD and catalyzes the reduction of methylenetetrahydrofolate to
35 methyltetrahydrofolate. The *metF* enzyme differs from

- 10 -

the mammalian enzyme in that it cannot be reduced by NADPH or NADH, and its activity is not allosterically regulated by S-adenosylmethionine. The native porcine enzyme is susceptible to tryptic cleavage between the

5 N-terminal 40 kDa domain and the C-terminal 37 kDa domain, and this cleavage results in the loss of allosteric regulation by adenosylmethionine, but does not result in loss of catalytic activity. Since the

10 homology between the bacterial and mammalian enzymes is within the N-terminal domain, this region must contain the flavin binding site and residues necessary to bind the folate substrate and catalyze its reduction. The domain structure of the human enzyme has not been

15 elucidated, although the human enzyme has been reported to have a molecular mass of 150 kDa and is likely to be a homodimer of 77 kDa.

We predict that the point of cleavage between the two domains lies between residues 351 and 374 of the human sequence, based on the localization of peptides obtained from the isolated domains of the porcine enzyme. This region, containing the highly-charged sequence KRREED, is predicted to have the highest hydrophilicity and surface probability of any region in the deduced human sequence.

20

25 The N-terminus of the porcine protein has been sequenced, and the region encoding this part of the protein is missing from the human cDNA. We estimate that this cDNA is missing only a few residues at the N-terminus, since the predicted molecular mass of the deduced sequence upstream of the putative cleavage

30 site (KRREED) is 40 kDa, and the measured molecular mass of the porcine N-terminal domain is also 40 kDa. When the bacterial, yeast and human sequences are aligned, the deduced human sequence contains an N-terminal

35 extension of 40 amino acids; we suspect that

- 11 -

this extension contains determinants for NADPH binding. Many pyridine nucleotide-dependent oxidoreductases contain such determinants at the N-terminus of the protein.

5 The C-terminus of the human sequence contains a peptide that is labeled when the protein is irradiated with ultraviolet light in the presence of tritiated AdoMet. The cDNA sequence we report here contains only about 7 kDa of the predicted 37 kDa mass of this
10 domain, indicating that this cDNA is truncated at the 3' terminus as well. A number of peptides from the C-terminal porcine domain have also not been detected. As might be expected, given that the prokaryotic enzymes do not appear to be allosterically regulated
15 by AdoMet, there are no significant homologies between the C-terminal region in this cDNA and the prokaryotic *metF* sequences. The alignment shown in Fig. 2 shows that the homologous sequences terminate just prior to the putative cleavage site of the human enzyme.

20

Chromosomal assignment

In situ hybridization to metaphase human chromosomes was used for localization of the human gene. The analysis of the distribution of 200 silver grains
25 revealed a significant clustering of grains 40 grains, in the p36.3-36.2 region of chromosome 1 ($p < 0.0001$), with the majority of grains, 25 grains, observed over lp36.3.

The isolation of the human cDNA has allowed us
30 to localize the gene to chromosome lp36.3. The observation of one strong signal on that chromosome with little background is highly suggestive of a single locus with no pseudogenes. Southern blotting of human DNA revealed fragments of approximately 10 kb, pre-

- 12 -

dicting a gene of average size, since this cDNA encodes approximately half of the coding sequence.

Additional cDNA sequences and constructs for expression analysis

5 A human colon carcinoma cDNA library (gift of Dr. Nicole Beauchemin, McGill University) was screened by plaque hybridization with the original 1.3 kb cDNA to obtain additional coding sequences. A cDNA of 2.2
10 kb was isolated, which contained 1.3 kb of overlapping sequence to the original cDNA and 900 additional bp at the 3' end (Fig. 6). The amino acid sequence is identical to that of the original cDNA for the overlapping segment (codons 1-415) except for codon 177 (ASP)
15 which was a GLY codon in the original cDNA. Analysis of 50 control chromosomes revealed an ASP codon at this position. The cDNA has an open reading frame of 1980 bp, 100 bp of 3' UTR and a poly A tail.

Sequencing was performed on both strands for
20 the entire cDNA. Additional 5' sequences (800 bp) were obtained from a human kidney cDNA library (Clontech) but these sequences did not contain additional coding sequences and were therefore used for the PCR-based mutagenesis only (as described below)
25 and not for the expression analysis. The two cDNAs (2.2 kb and 800 bp) were ligated using the *EcoRI* site at bp 199 and inserted into the Bluescript™ vector (Stratagene). The 2.2 kb cDNA was subcloned into the expression vector pTrc99A (Pharmacia) using the *NcoI*
30 site at bp 11 and the *XbaI* site in the polylinker region of both the Bluescript™ and the pTrc99A vectors. Sequencing was performed across the cloning sites to verify the wild-type construct.

UTILITY OF INVENTION IN IDENTIFICATION OF MUTATIONS

I. Identification of first two mutations in severe MTHFR deficiency

Total RNA of skin fibroblasts from MTHFR-deficient patients was reverse-transcribed and amplified by PCR for analysis by the single strand conformation polymorphism (SSCP) method (Orita, M. et al., *Genomics*, 1989, 5:8874-8879). Primers were designed to generate fragments of 250-300 bp and to cover the available cDNA sequences with small regions of overlap for each fragment at both ends. The first mutation identified by SSCP was a C to T substitution at bp 559 in patient 1554; this substitution converted an arginine codon to a termination codon (Fig. 3A). Since the mutation abolished a *FokI* site, restriction digestion was used for confirmation of the change and for screening additional patients for this mutation; a second patient (1627) was identified in this manner (Fig. 3B). The SSCP pattern for patient 1554 and the restriction digestion pattern for both patients was consistent with a homozygous mutant state or with a genetic compound consisting of the nonsense mutation with a second mutation that did not produce any detectable RNA (null allele). Studies in the parents are required for confirmation.

The second substitution (Fig. 4A) was a G to A transition at bp 482 in patient 1834 that converted an arginine into a glutamine residue. The substitution created a *PstI* site which was used to verify the substitution and to identify a second patient (1863) with this change (Fig. 4B). The SSCP analysis and the restriction digestion pattern were consistent with a heterozygous state for both patients. The arginine codon affected by this change is an evolutionarily-conserved residue, as shown in Fig. 2. This observa-

- 14 -

tion, in conjunction with the fact that the codon change is not conservative, makes a strong argument that the substitution is a pathologic change rather than a benign polymorphism. Furthermore, 35 controls
5 (of similar ethnic background to that of the probands) were tested for this substitution by Southern blotting of PstI-digested DNA; all were negative.

The family of patient 1834 was studied. The symptomatic brother and the mother of the proband were
10 all shown to carry this substitution, whereas the father was negative for the change (Fig. 4B). In the family of 1863, the mother of the proband was shown to be a carrier, while the father and an unaffected brother were negative.

15

Cell lines

Cell line 1554 is from a Hopi male who was admitted at age three months with homocystinuria, seizures, dehydration, corneal clouding, hypotonia and
20 candida sepsis. Folate distribution in cultured fibroblasts showed a *Pediococcus cerivisiae*/*Lactobacillus casei*(PC/LC) ratio of 0.52 (Control 0.14). There was no measurable methylenetetrahydrofolate reductase (MTHFR) activity
25 (Control values = 9.7 and 15.1 nmoles/h/mg protein; residual activity after treatment of control extracts at 55°C for 20 min. = 28% and 31%).

Cell line 1627 is from a Choctaw male who presented with poor feeding, apnea, failure to thrive,
30 dehydration and homocystinuria at five weeks of age. He was subsequently found to have superior sagittal sinus thrombosis and hydrocephalus. The PC/LC ratio was 0.61 and the specific activity of MTHFR was 0.1 nmoles/h/mg protein. There is consanguinity in that

- 15 -

the maternal and paternal grandmothers are thought to be "distantly related".

Cell line 1779 is from a French Canadian male with homocystinuria who first had limb weakness, inco-
5 ordination, paresthesiae, and memory lapses at age 15 years, and was wheelchair-bound in his early twenties. His brother (cell line 1834) also has homocystinuria, but is 37 years old and asymptomatic. Specific activ-
ity of MTHFR was 0.7 and 0.9 nmole/h/mg protein for
10 1779 and 1834, respectively; the residual activity after heat treatment at 55°C was 0.9% and 0% for 1779 and 1834, respectively.

Cell line 1863 is from a white male who was diagnosed at age 21 years because of a progressive
15 gait disturbance, spasticity, cerebral white matter degeneration, and homocystinuria. He had a brother who died at age 21 years of neurodegenerative disease. Specific activity of MTHFR in fibroblast extracts was
1.76 nmoles/h/mg protein and the residual enzyme ac-
20 tivity after treatment at 55°C was 3.6%.

Mutation analysis

Primers were designed from the cDNA sequence to generate 250-300 bp fragments which overlapped 50-
25 75 bp at each end. The primer pairs were used in reverse transcription-PCR of 5µg patient total fibroblast RNA. The PCR products were analyzed by a non-isotopic rapid SSCP protocol (PhastSystem™, Pharmacia), which uses direct silver staining for detection
30 of single strands. Any PCR products from patients showing a shift on SSCP gels were purified by NuSieve (FMC Bioproducts) and sequenced directly (Cycle Sequencing™ kit, GIBCO) to identify the change. If the change affected a restriction site, then a PCR
35 product was digested with the appropriate restriction endonuclease and analyzed on polyacrylamide gels. To

- 16 -

screen for the Arg to Gln mutation in controls, 5 µg of *Pst*I-digested DNA was run on 0.8% agarose gels and analyzed by Southern blotting using the radiolabelled cDNA by standard techniques.

5

II. Seven additional mutations at the methylene-tetrahydrofolate reductase (MTHFR) locus with genotype: phenotype correlations in severe MTHFR deficiency

10

It is reported hereinbelow the characterization of 7 additional mutations at this locus: 6 missense mutations and a 5' splice site defect which activates a cryptic splice site in the coding sequence. We also present a preliminary analysis of the relationship between genotype and phenotype for all 9 mutations identified thus far at this locus. A nonsense mutation and 2 missense mutations (proline to leucine and threonine to methionine) in the homozygous state are associated with extremely low activity (0-3%) and onset of symptoms within the first year. Other missense mutations (arginine to cysteine and arginine to glutamine) are associated with higher enzyme activity and later onset of symptoms.

20

7 additional mutations at the MTHFR locus are described and the association between genotype, enzyme activity, and clinical phenotype in severe MTHFR deficiency is examined.

30 **Patient description**

The clinical and laboratory findings of the patients have been reported in the published literature. Residual MTHFR activity was previously measured in cultured fibroblasts at confluence.

35

Patient 354, an African-American girl, was diagnosed at age 13 years with mild mental retardation. Her sister, patient 355 was diagnosed at age 15

- 17 -

years with anorexia, tremor, hallucinations and progressive withdrawal. In patient 354, residual MTHFR activity was 19% and in her sister, 355, it was 14% of control values. The residual activity after heating
5 had equivalent thermal stability to control enzyme.

Patient 1807, a Japanese girl whose parents are first cousins, had delayed walking and speech until age 2 years, seizures at age 6 years and a gait disturbance with peripheral neuropathy at age 16 years.
10 Residual activity of MTHFR was 3% and the enzyme was thermolabile.

Patient 735, an African-Indian girl, was diagnosed at age 7 months with microcephaly, progressive deterioration of mental development, apnea and coma.
15 Residual activity of MTHFR was 2% of control levels. Thermal properties were not determined.

Patient 1084, a Caucasian male, was diagnosed at age 3 months with an infantile fibrosarcoma. He was found to be hypotonic and became apneic. He died
20 at the age of 4 months. Residual activity of MTHFR was not detectable. Thermal properties were not determined.

Patient 356, the first patient reported with MTHFR deficiency, is an Italian-American male who presented at age 16 years with muscle weakness, abnormal gait and flinging movements of the upper extremities.
25 MTHFR residual activity was 20% of control values; activity was rapidly and exponentially inactivated at 55°.

Patient 458, a Caucasian male, was diagnosed at age 12 years with ataxia and marginal school performance. Residual MTHFR activity was approximately 10%, and the activity was thermolabile.
30

Patient 1396, a Caucasian female, was described
35 as clumsy and as having a global learning disorder in

- 18 -

childhood. At age 14 years, she developed ataxia, foot drop, and inability to walk. She developed deep vein thrombosis and bilateral pulmonary emboli. Residual activity of MTHFR was 14% and the enzyme was thermolabile.

Genomic structure and intronic primers

Exon nomenclature is based on available cDNA sequence in Goyette et al. (*Nature Genetics*, 1994, 7:195-200). Exon 1 has been arbitrarily designated as the region of cDNA from bp 1 to the first intron. Identification of introns was performed by amplification of genomic DNA using cDNA primer sequences. PCR products that were greater in size than expected cDNA sizes were sequenced directly.

Mutation detection

Specific exons (see Table 1 for primer sequences) were amplified by PCR from genomic DNA and analyzed by the SSCP protocol. SSCP was performed with the Phastgel™ system (Pharmacia), a non-isotopic rapid SSCP protocol, as previously described (Goyette P et al., *Nature Genetics*, 1994, 7:195-200), or with ³⁵S-labeled PCR products run on 6% acrylamide: 10% glycerol gels at room temperature (6 watts, overnight). In some cases, the use of restriction endonucleases, to cleave the PCR product before SSCP analysis, enhanced the detection of band shifts. PCR fragments with altered mobility were sequenced directly (GIBCO, Cycle Sequencing™ kit). If the sequence change affected a restriction endonuclease site, then the PCR product was digested with the appropriate enzyme and analyzed by PAGE. Otherwise, allele-specific oligonucleotide (ASO) hybridization was performed on a dot blot of the PCR-amplified exon.

Table 1
PCR Primers for DNA amplification and mutation
analysis of MTHFR

| Exon | Primer Type | Primer Sequence (5'→3') | Location | Fragment Size (bp) |
|------|-------------|-------------------------|----------|--------------------|
| 1 | Sense | AGCCTCAACCCCTGCTTGGAGG | C | 271 |
| | Antisense | TGACAGTTTGCTCCCCAGGCAC | I | |
| 4 | Sense | TGAAGGAGAAGGTGTCTGCCGGA | C | 198 |
| | Antisense | AGGACGGTGCGGTGAGAGTGG | I | |
| 5 | Sense | CACTGTGGTTGGCATGGATGATG | I | 392 |
| | Antisense | GGCTGCTCTTGGACCCTCCTC | I | |
| 6 | Sense | TGCTCCGGCTCCCTCTAGCC | I | 251 |
| | Antisense | CCTCCCGCTCCCAAGAACAAG | I | |

5

Table 2
Summary of genotypes, enzyme activity, age at onset,
and background of patients with MTHFR deficiency

| Patient ^a | BPChanges ^b | Amino acid changes | % Activity | Age at Onset | Background |
|----------------------|------------------------|--------------------|------------|------------------------|---------------------------|
| 1807 | C764T/C764T | Pro→Leu/Pro→Leu | 3 | within 1st year | Japanese |
| 735 | C692T/C692T | Thr→Met/Thr→Met | 2 | 7 months | African Indian |
| 1084 | C692T/C692T | Thr→Met/Thr→Met | 0 | 3 months | Caucasian |
| 1554 | C559T/C559T | Arg→Ter/Arg→Ter | 0 | 1 month | Native American (Hopi) |
| 1627 | C559T/C559T | Arg→Ter/Arg→Ter | 1 | 1 month | Native American (Choctaw) |
| 356 | C985T/C985T | Arg→Cys/Arg→Cys | 20 | 18 yrs | Italian American |
| 458 | C1015T/G167A | Arg→Cys/Arg→Gln | 10 | 11 yrs | Caucasian |
| 1396 | C1081T/G167A | Arg→Cys/Arg→Gln | 14 | 14 yrs | Caucasian |
| 1779 ^c | G482A/? | Arg→Gln/? | 6 | 15 yrs | French Canadian |
| 1834 ^c | G482A/? | Arg→Gln/? | 7 | Asymptomatic at 37 yrs | French Canadian |
| 1863 | G482A/? | Arg→Gln/? | 14 | 21 yrs | Caucasian |
| 354 ^d | 792 + 1G→A/? | 5' splice site/? | 19 | 13 yrs | African American |
| 355 ^d | 792 + 1G→A/? | 5' splice site/? | 14 | 11 yrs | African American |

10

^a Patients 1554, 1627, 1779, 1834 and 1863 were previously reported by Goyette et al. (1994).

^b ? = unidentified mutation.

^c Patients 1779 and 1834 are sibs.

^d Patients 354 and 355 are sibs.

- 20 -

(1) 5' splice site mutation

Amplification of cDNA, bp 653-939, from reverse-transcribed total fibroblast RNA revealed 2 bands in sisters 354 and 355: a smaller PCR fragment (230 bp) in addition to the normal 287 bp allele (Fig. 8A). Fig. 8A is the PAGE analysis of amplification products of cDNA bp 653-939, from reverse transcribed RNA. Controls have the expected 287 bp fragment while patients 354 and 355 have an additional 230 bp fragment. Sequencing of the smaller fragment identified a 57 bp in-frame deletion which would remove 19 amino acids (Fig. 8B). Fig. 8B is the direct sequencing of the PCR products from patient 354. The 57 bp deletion spans bp 736-792 of the cDNA. An almost perfect 5' splice site (boxed) is seen at the 5' deletion breakpoint. Analysis of the sequence at the 5' deletion breakpoint in the undeleted fragment revealed an almost perfect 5' splice site consensus sequence (AG/gcatgc). This observation suggested the presence of a splicing mutation in the natural 5' splice site that might activate this cryptic site, to generate the deleted allele. The sequence following the deletion breakpoint, in the mutant allele, corresponded exactly to the sequence of the next exon. Amplification of genomic DNA, using the same amplification primers as those used for reverse-transcribed RNA, generated a 1.2 kb PCR product indicating the presence of an intron. Direct sequencing of this PCR fragment in patient 354 identified a heterozygous G→A substitution in the conserved GT dinucleotide of the intron at the 5' splice site (Fig. 8C). Fig. 8C is the sequencing of the 5' splice site in control and patient 354. The patient carries a heterozygous G→A substitution in the 5' splice site (boxed). Intronic sequences are in

- 21 -

lower case. This substitution abolished a *Hph*I restriction endonuclease site which was used to confirm the mutation in the 2 sisters (Fig. 8D). Fig. 8D is the *Hph*I restriction endonuclease analysis on PCR products of DNA for exon 4 of patients 354 and 355, and of 3 controls (C). The 198 bp PCR product has 2 *Hph*I sites. The products of digestion for the control allele are 151, 24 and 23 bp. The products of digestion for the mutant allele are 175 and 23 bp due to the loss of a *Hph*I site. The fragments of 24 and 23 bp have been run off the gel.

(2) Patients with homozygous coding substitutions

SSCP analysis of exon 4 for patient 1807 revealed an abnormally-migrating fragment, which was directly sequenced to reveal a homozygous C→T substitution (bp 764) converting a proline to a leucine residue. This change creates a *Mnl*I restriction endonuclease site, which was used to confirm the homozygous state of the mutation (Fig. 9A). Fig. 9A is the *Mnl*I restriction analysis of exon 4 PCR products for patient 1807 and 3 controls (C). Expected fragments: control allele, 90, 46, 44, 18 bp; mutant allele, 73, 46, 44, 18, 17 bp. An additional band at the bottom of the gel is the primer. Fifty independent control Caucasian chromosomes and 12 control Japanese chromosomes were tested by restriction analysis; all were negative for this mutation. Homozygosity in this patient is probably due to the consanguinity of the parents.

Patients 735 and 1084 had the same mutation in exon 4, in a homozygous state: a C→T substitution (bp 692) which converted an evolutionarily-conserved threonine residue to a methionine residue, and abolished a *Nla*III restriction endonuclease site. Allele-

- 22 -

specific oligonucleotide hybridization to amplified exon 4 (Figs. 10A and 10B) was used to confirm the mutation in these 2 patients and to screen 60 independent chromosomes, all of which turned out to be negative. Fig. 10A is the hybridization of mutant oligonucleotide (692T) to exon 4 PCR products from patients 735, 1084 and 30 controls. Only DNA from patients 735 and 1084 hybridized to this probe. Fig. 10B is the hybridization of normal oligonucleotide (692C) to stripped dot blot from A. All control DNAs hybridized to this probe.

Patient 356 showed a shift on SSCP analysis of exon 5. Direct sequencing revealed a homozygous C→T substitution (bp 985) which converted an evolutionarily-conserved arginine residue to cysteine; the substitution abolished an *AciI* restriction endonuclease site. This was used to confirm the homozygous state of the mutation in patient 356 (Fig. 9B) and its presence in the heterozygous state in both parents. Fifty independent control chromosomes, tested in the same manner, were negative for this mutation. Fig. 9B is the *AciI* restriction analysis of exon 5 PCR products for patient 356, his father (F), his mother (M), and 3 controls (C). Expected fragments: control allele, 129, 105, 90, 68 bp; mutant allele, 195, 129, 68 bp.

(3) Patients who are genetic compounds

Patient 458 is a compound heterozygote of a mutation in exon 5 and a mutation in exon 1. The exon 5 substitution (C→T at bp 1015) resulted in the substitution of a cysteine residue for an arginine residue; this abolished a *HhaI* restriction endonuclease site, which was used to confirm the mutation in patient 458 (Fig. 9C) and to show that 50 control chromosomes were negative. Fig. 9C is the *HhaI*

restriction analysis of exon 5 PCR products for patient 458 and 4 controls (C). Expected fragments: control allele, 317 and 75 bp; mutant allele 392 bp. The 75 bp fragment is not shown in Fig. 9C. The

5 second mutation was a heterozygous G→A substitution (bp 167) converting an arginine to a glutamine residue. Allele-specific oligonucleotide hybridization to amplified exon 1 confirmed the heterozygous state of this mutation in patient 458 and identified a second

10 patient (1396) carrying this mutation also in the heterozygous state (Figs. 10C and 10D). Fig. 10C is the hybridization of mutant oligonucleotide (167A) to exon 1 PCR products from patients 458, 1396 and 31 controls. Fig. 10D is the hybridization of normal

15 oligonucleotide (167G) to stripped dot blot from C. None of the 62 control chromosomes carried this mutation.

The second mutation in patient 1396 was identified in exon 6: a heterozygous C→T substitution (bp

20 1081) that converted an arginine residue to a cysteine residue, and abolished a *HhaI* restriction endonuclease site. Restriction analysis confirmed the heterozygous substitution in 1396 (Fig. 9D) and showed that 50 control chromosomes were negative. Fig. 9D is the *HhaI*

25 restriction analysis of exon 6 PCR products for patient 1396 and 2 controls (C). Expected fragments: control allele, 152, 86, 13 bp; mutant allele 165, 86 bp. The 13 bp fragment has been run off the gel.

30 (4) Additional sequence changes

HhaI analysis of exon 6, mentioned above, revealed a DNA polymorphism. This change is a T→C substitution at bp 1068 which does not alter the amino acid (serine), but creates a *HhaI* recognition site. T

35 at bp 1068 was found in 9% of tested chromosomes.

- 24 -

Sequence analysis identified 2 discrepancies with the published cDNA sequence: a G→A substitution at bp 542 which converts the glycine to an aspartate codon, and a C→T change at bp 1032 which does not alter the amino acid (threonine). Since all DNAs tested (>50 chromosomes) carried the A at bp 542 and the T at bp 1032, it is likely that the sequence of the original cDNA contained some cloning artifacts.

10 Genotype:phenotype correlation

Table 2 summarizes the current status of mutations in severe MTHFR deficiency. In 8 patients, both mutations have been identified; in 5 patients (3 families), only 1 mutation has been identified. Overall the correlation between the genotype, enzyme activity, and phenotype is quite consistent. Five patients, with onset of symptoms within the first year of life, had $\leq 3\%$ of control activity. Three of these patients had missense mutations in the homozygous state: two patients with the threonine to methionine substitution (C692T) and one patient with the proline to leucine substitution (C764T). The nonsense mutation (C559T) in the homozygous state in patients 1554 and 1627 (previously reported in Goyette P et al., *Nature Genetics*, 1994, 7:195-200) is also associated with a neonatal severe form, as expected.

The other patients in Table 2 had $\geq 6\%$ of control activity and onset of symptoms within or after the 2nd decade of life; the only exception is patient 1834, as previously reported (Goyette P et al., *Nature Genetics*, 1994, 7:195-200). The three patients (356, 458 and 1396) with missense mutations (G167A, C985T, C1015T and C1081T) are similar to those previously reported (patients 1779, 1834 and 1863) who had an arginine to glutamine substitution and a second

- 25 -

unidentified mutation (Goyette P et al., *Nature Genetics*, 1994, 7:195-200). The sisters with the 5' splice mutation and an unidentified second mutation also had levels of activity in the same range and onset of
5 symptoms in the second decade, but the activity is likely due to the second unidentified allele.

Discussion

The patients come from diverse ethnic back-
10 grounds. Although patients 1554 and 1627 are both Native Americans, the mutations occur on different haplotypes, suggesting recurrent mutation rather than identity by descent. Since the substitution occurs in a CpG dinucleotide, a "hot spot" for mutation, recur-
15 rent mutation is a reasonable hypothesis. It is difficult to assess whether some mutations are population-specific since the numbers are too small at the present time.

MTHFR deficiency is the most common inborn
20 error of folate metabolism, and a major cause of hereditary homocysteinemia. The recent isolation of a cDNA for MTHFR has permitted mutational analysis at this locus, with the aims of defining important domains for the enzyme and of correlating genotype
25 with phenotype in MTHFR-deficient patients.

Our definition of a disease-causing substitution, as distinct from a benign polymorphism, is based on 3 factors: (1) absence of the change in at least 50 independent control chromosomes; (2) presence of the
30 amino acid in the bacterial enzyme, attesting to its evolutionary significance and (3) whether the change in amino acid is conservative. Although expression of the substitutions is required to formally prove that they are not benign, the criteria above allow us to

- 26 -

postulate that the changes described in this report are likely to affect activity.

The 7 mutations described here (6 single amino acid substitutions and a 5' splice site mutation) bring the total to 9 mutations identified thus far in severe MTHFR deficiency and complete the mutation analysis for 8 patients. The identification of each mutation in only one or two families points to the striking degree of genetic heterogeneity at this locus. Seven of the 9 mutations are located in CpG dinucleotides, which are prone to mutational events.

5' splice site mutation

The G→A substitution at the GT dinucleotide of the 5' splice site in patients 354 and 355 results in a 57bp in-frame deletion of the coding sequence, which should delete 19 amino acids of the protein. This deletion occurs as a result of the activation of a cryptic 5' splice site (AG/gc) even though this cryptic site does not have a perfect 5' splice site consensus sequence (AG/gt). However, GC (instead of GT) as the first 2 nucleotides of an intron have been reported in several naturally-occurring splice sites, such as in the genes for human prothrombin and human adenine phosphoribosyltransferase and twice within the gene for the largest subunit of mouse RNA polymerase II. The remaining nucleotides of the cryptic site conform to a normal splice site consensus sequence with its expected variations (A₆₀G₇₉/G₁₀₀T₁₀₀A₅₉A₇₁G₈₂T₅₀). It is unlikely that the deleted enzyme resulting from this aberrantly-spliced mRNA would have any activity; 8 of the 19 deleted amino acids are conserved in the *E. coli* enzyme. Although the 2 patients show residual enzyme activity in the range of 20% of controls, the activity is prob-

- 27 -

ably due to the unidentified second allele in these patients.

6 missense mutations

5 The Arg→Cys substitution (C1081T) in patient 1396 is within a hydrophilic sequence previously postulated to be the linker region between the catalytic and regulatory domains of MTHFR (Goyette P et al., *Nature Genetics*, 1994, 7:195-200). These 2 domains
10 are readily separable by mild trypsinization of the porcine enzyme. The linker domain, a highly-charged region, is likely to be located on the outside surface of the protein and therefore more accessible to proteolysis. Because the Arg→Cys substitution converts a
15 charged hydrophilic residue to an uncharged polar residue, it cannot be considered a conservative change, and could affect the stability of the enzyme.

 The 2 Arg→Cys substitutions identified in patients 356 and 458 (C985T and C1015T, respectively)
20 may be involved in binding the FAD cofactor. Previous work in the literature showed that heating fibroblast extracts at 55°, in the absence of the FAD cofactor, inactivated MTHFR completely. The addition of FAD to the reaction mixture before heat inactivation restored some enzyme activity to control extracts
25 and to extracts from some patients, while the extracts of patients 356 and 458 were unaffected. Based on these observations, it was suggested that these 2 patients had mutations affecting a region of the protein involved in binding FAD. The 2 mutations are
30 found in close proximity to each other, within 11 amino acids. In patient 356, the Arg residue is evolutionarily-conserved in *E. coli* and is found in a stretch of 9 conserved amino acids, suggesting a
35 critical role for this residue; the altered Arg resi-

due in patient 458 is not evolutionarily-conserved. Crystal structure analysis of medium chain acyl-CoA dehydrogenase (MCAD), a flavoprotein, has defined critical residues involved in the binding of FAD. Two consecutive residues of the MCAD protein, Met165 and Trp166, involved in interactions with FAD, can also be identified in MTHFR, 3 and 4 amino acids downstream, respectively, from the Arg residue altered in patient 458.

10 The Thr→Met substitution (C692T), is found in a region of high conservation with the *E. coli* enzyme and in a region of good homology with human dihydrofolate reductase (DHFR) (Fig. 11). In Fig. 11, = is identity; • is homology; and ◇ is identity to bovine DHFR enzyme. An asterisk (*) indicates location of Thr→Met substitution. Considering the early-onset phenotype of the patients, one can assume that the threonine residue is critical for activity or that it contributes to an important domain of the protein.

15 This region of homology in DHFR contains a residue, Thr136, which has been reported to be involved in folate binding. This Thr residue in DHFR aligns with a Ser residue in MTHFR, an amino acid with similar biochemical properties. The Thr → Met substitution is

20 located 8 amino acids downstream from this Ser codon, in the center of the region of homology between the 2 enzymes. We therefore hypothesize that the Thr → Met substitution may alter the binding of the folate substrate.

30 The G167A (Arg → Gln) and C764T (Pro → Leu) substitutions both affect non-conserved amino acids. Their importance in the development of MTHFR deficiency cannot be determined at the present time. All the mutations identified thus far are located in the

35 5' end of the coding sequence, the region thought to

encode the catalytic domain of MTHFR. Mutation analysis has been useful in beginning to address the structure: function properties of the enzyme as well as to understand the diverse phenotypes in severe MTHFR
5 deficiency.

III. Identification of A→V mutation

SSCP analysis and direct sequencing of PCR fragments were used to identify a C to T substitution
10 at bp 677, which converts an alanine residue to a valine residue (Fig. 5A). The primers for analysis of the A→V change are: 5'-TGAAGGAGAA GGTGTCTGCG GGA-3' (exonic) and 5'-AGGACGGTGC GGTGAGAGTG-3'(intronic); these primers generate a fragment of 198 bp. Fig. 5A
15 depicts the sequence of two individuals, a homozygote for the alanine residue and a homozygote for the valine residue. The antisense strands are depicted. This alteration creates a *HinfI* site (Fig. 5B), which was used to screen 114 unselected French Canadian
20 chromosomes; the allele frequency of the substitution was .38. The substitution creates a *HinfI* recognition sequence which digests the 198 bp fragment into a 175 bp and a 23 bp fragment; the latter fragment has been run off the gel. Fig. 5B depicts the three possible
25 genotypes. The frequency of the 3 genotypes were as follows: -/-, 37%; +/-, 51%; and +/+, 12% (the (+) indicates the presence of the *HinfI* restriction site and a valine residue).

The alanine residue is conserved in porcine
30 MTHFR, as well as in the corresponding *metF* and *stymetF* genes of *E. coli* and *S. typhimurium*, respectively. The strong degree of conservation of this residue, and its location in a region of high homology with the bacterial enzymes, alluded to its importance
35 in enzyme structure or function. Furthermore, the

frequency of the (+/+) genotype was consistent with the frequency of the thermolabile MTHFR variant implicated in vascular disease.

5 Clinical material

To determine the frequency of the A→V mutation, DNA from 57 individuals from Quebec was analyzed by PCR and restriction digestion. The individuals, who were all French Canadian, were not examined clinically or biochemically.

The 40 individuals analyzed in Table 3 had been previously described in Engbersen et al. (*Am. J. Hum. Genet.*, 1995, 56:142-150). Of the 13 cardiovascular patients, 8 had cerebrovascular arteriosclerosis and 5 had peripheral arteriosclerosis. Five had thermolabile MTHFR while 8 had thermostable MTHFR (greater than 33% residual activity after heating). Controls and patients were all Dutch-Caucasian, between 20-60 years of age. None of these individuals used vitamins which could alter homocysteine levels. Enzyme assays and homocysteine determinations were also reported by Engbersen et al. (*Am. J. Hum. Genet.*, 1995, 56:142-150).

Table 3

Correlation between MTHFR genotype and enzyme activity, thermolability and plasma homocysteine level

5

| | -/- n=19 | +/- n=9 | +/+ n=12 |
|--|--|---------------------------|--|
| specific activity ^{a,b} (nmol CH ₂ O/mg.protein/hr) | 22.9 ± 1.7 (11.8 - 33.8) | 15.0 ± 0.8 (10.2-18.8) | 6.9 ± 0.6 (2.6-10.2) |
| residual activity after heating ^{a,b} (%) | 66.8 ± 1.5 (55-76) | 56.2 ± 2.8 (41-67) | 21.8 ± 2.8 (10-35) |
| plasma homocysteine ^{a,c} (μM)(after fasting) | 12.6 ± 1.1 (7-21) | 13.8 ± 1.0 (9.6-20) | 22.4 ± 2.9 (9.6-42) |
| plasma homocysteine ^{a,c} (μM)(post-methionine load) | 41.3 ± 5.0 ^d (20.9 -110) | 41 ± 2.8 (29.1-54) | 72.6 ± 11.7 ^e (24.4-159) |

^a one-way anova p<.01

^b paired t test for all combinations p<.01

^c paired t test p<.05 for +/+ group versus +/- group or -/- group; p>.05 for +/- versus -/- group.

10 ^d n=18 for this parameter

^e n=11 for this parameter

Enzyme activity and plasma homocysteine were determined as previously reported. Each value represents mean ± standard error. The range is given in parentheses below the mean.

15

Correlation of A→V mutation with altered MTHFR function

A genotypic analysis was performed and enzyme activity and thermolability were measured in a total of 40 lymphocyte pellets from patients with premature vascular disease and controls. 13 vascular patients were selected from a previous study (Engbersen et al., *Am. J. Hum. Genet.*, 1995, 56:142-150), among which 5 were considered to have thermolabile MTHFR. From a large reference group of 89 controls, all 7 individuals who had thermolabile MTHFR were studied, and an additional 20 controls with normal MTHFR were selected from the same reference group. Table 3 documents the relationship between genotypes and spe-

30

cific enzyme activity, thermolability and plasma homocysteine level. The mean MTHFR activity for individuals homozygous for the substitution (+/+) was approximately 30% of the mean activity for (-/-) individuals, homozygous for the alanine residue. Heterozygotes had a mean MTHFR activity that was 65% of the activity of (-/-) individuals; this value is intermediate between the values for (-/-) and (+/+) individuals. The ranges of activities showed some overlap for the heterozygous and (-/-) genotypes, but homozygous (+/+) individuals showed virtually no overlap with the former groups. A one-way analysis of variance yielded a p value <.0001; a pairwise Bonferroni t test showed that all three genotypes were significantly different with p<0.01 for the three possible combinations.

The three genotypes were all significantly different (p<.01) with respect to enzyme thermolability. The mean residual activity after heat inactivation for 5 minutes at 46° was 67%, 56% and 22% for the (-/-), (+/-) and (+/+) genotypes, respectively. While the degree of thermolability overlaps somewhat for (-/-) individuals and heterozygotes, individuals with two mutant alleles had a distinctly lower range. Every individual with the (+/+) genotype had residual activity <35% after heating, and specific activity <50% of that of the (-/-) genotype.

Total homocysteine concentrations, after fasting and 6 hours after methionine loading, were measured in plasma by high performance liquid chromatography using fluorescence detection. Fasting homocysteine levels in (+/+) individuals were almost twice the value for (+/-) and (-/-) individuals. The differences among genotypes for plasma homocysteine were maintained when homocysteine was measured following 6 hours of methionine loading. A one-way anova yielded

- 33 -

a $p < .01$ for the fasting and post-methionine homocysteine levels. A pairwise Bonferroni t test showed that only homozygous mutant individuals had significantly elevated homocysteine levels ($p < .05$).

5

PCR-based mutagenesis for expression of A→V mutation in vitro

PCR-based mutagenesis, using the cDNA-containing Bluescript™ vector as template, was used to create the A to V mutation. Vent™ polymerase (NEB) was used to reduce PCR errors. The following primers were used: primer 1, bp -200 to -178, sense; primer 2, bp 667 to 687, antisense, containing a mismatch, A, at bp 677; primer 3, 667 to 687, sense, containing a mismatch, T, at bp 677; primer 4, bp 1092 to 1114, antisense. PCR was performed using primers 1 and 2 to generate a product of 887 bp, and using primers 3 and 4 to generate a product of 447 bp. The two PCR fragments were isolated from a 1.2% agarose gel by Gene-clean™ (BIO 101). A final PCR reaction, using primers 1 and 4 and the first 2 PCR fragments as template, was performed to generate a 1.3 kb band containing the mutation. The 1.3 kb fragment was digested with NcoI and MscI, and inserted into the wild-type cDNA-containing expression vector by replacing the sequences between the NcoI site at bp 11 and the MscI site at bp 943. The entire replacement fragment and the cloning sites were sequenced to verify that no additional changes were introduced by PCR.

30

Expression analysis of wild-type and mutagenized cDNA

Overnight cultures of JM105™ containing vector alone, vector + wild-type MTHFR cDNA, or vector + mutagenized cDNA were grown at 37° C. in 2 x YT media with .05 mg/ml ampicillin. Fresh 10 ml. cultures of

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- 34 -

each were inoculated with approximately 50 μ L of over-night cultures for a starting O.D. of 0.05, and were grown at 37° C to an O.D. of 1 at 420 nm. Cultures were then induced for 2 hrs. with 1 mM IPTG and pel-
5 letted. The cells were resuspended in TE buffer with 2 μ g/ml aprotinin and 2 μ g/ml leupeptin (3.5 x wet weight of cells). Cell suspensions were sonicated on ice for 3 x 15 sec. to break open cell membranes and then centrifuged for 30 mins. at 4°C. to pellet cell
10 debris and unlysed cells. The supernatant was removed and assayed for protein concentration with the Bio-Rad™ protein assay. Western analysis was performed using the Amersham ECL™ kit according to the instructions of the supplier, using antiserum generated against
15 purified porcine liver MTHFR. Enzymatic assays were performed by established procedures; thermolability was assessed by pre-treating the extracts at 46°C. for 5 mins. before determining activity. Specific activities (nmol formaldehyde/hr./mg. protein) were calculated for the 2 cDNA-containing constructs after subtraction of the values obtained with vector alone (to subtract background *E. coli* MTHFR activity).

The MTHFR cDNA (2.2 kb) (Fig. 6) has an open reading frame of 1980 bp, predicting a protein of 74.6
25 kDa. The purified porcine liver enzyme has been shown to have subunits of 77 kDa. Western analysis (Fig. 7A) of several human tissues and of porcine liver has revealed a polypeptide of 77 kDa in all the studied tissues, as well as an additional polypeptide
30 of approximately 70 kDa in human fetal liver and in porcine liver, suggesting the presence of isozymes. Two μ g of bacterial extract protein was used for lanes 1-3. The tissues (lanes 4-8) were prepared by homogenization in .25M sucrose with protease
35 inhibitors (2 μ g/ml each of aprotinin and leupeptin),

- 35 -

followed by sonication (3 x 15 sec.) on ice. The extracts were spun for 15 min. in a microcentrifuge at 14,000 g and 100 µg of supernatant protein was used for Western analysis. h=human; p=porcine.

5 The wild-type cDNA and a mutagenized cDNA, containing the A→V substitution, were expressed in *E. coli* to yield a protein of approximately 70 kDa (Fig. 7A), which co-migrates with the smaller polypeptide mentioned above. Treatment of extracts at 46°C
10 for 5 minutes revealed that the enzyme containing the substitution was significantly more thermolabile than the wild-type enzyme ($p < .001$; Fig. 7B). Two separate experiments (with 3-4 replicates for each construct for each experiment) were performed to measure thermo-
15 stable activity of the wild-type MTHFR and mutagenized MTHFR A→V cDNAs. The values shown represent mean \pm standard error for each experiment, as % of residual activity after heating. The means of the specific activities before heating (expressed as nmol formalde-
20 hyde/hr./mg. protein) were as follows: Exp. 1, 3.8 and 5.3 for MTHFR and MTHFR A→V, respectively; Exp. 2, 6.2 and 7.5 for MTHFR and MTHFR A→V, respectively. The expression experiments were not designed to measure differences in specific activity before heating, since
25 variation in efficiencies of expression could contribute to difficulties in interpretation. Curiously though, the specific activity for the mutant construct was higher in both experiments. It is possible that the mutant protein has increased stability in *E. coli*,
30 or that inclusion bodies in our extracts contributed to differences in recovery of properly-assembled enzyme.

 These studies have identified a common substitution in the MTHFR gene which results in thermolability in vitro and in vivo. The mutation, in the
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- 36 -

heterozygous or homozygous state, correlates with reduced enzyme activity and increased thermolability of MTHFR in lymphocyte extracts. A significant elevation in plasma homocysteine was observed in individuals who were homozygous for the mutation. Statistically-significant differences for homocysteine levels were not observed between heterozygotes and (-/-) individuals; this observation is not surprising, since plasma homocysteine can be influenced by several environmental factors, including intake of folate, vitamin B₁₂, vitamin B₆, and methionine, as well as by genetic variation at other loci, such as the cystathionine- β -synthase gene.

The alanine to valine substitution conserves the hydrophobicity of the residue and is associated with small changes in activity, in contrast to non-conservative changes, such as the previously-reported arginine to glutamine change in MTHFR, which is associated with a greater decrease in enzyme activity and severe hyperhomocysteinemia. The alanine residue is situated in a region of homology with the bacterial metF genes. We have also observed the same region of homology in the human dihydrofolate reductase (DHFR) gene (Fig. 11), although the alanine residue itself is not conserved; this region of amino acids 130-149 of DHFR contains T136 which has been implicated in folate binding in an analysis of the crystal structure of recombinant human DHFR. It is tempting to speculate that this region in MTHFR is also involved in folate binding and that the enzyme may be stabilized in the presence of folate. This hypothesis is compatible with the well-documented influence of folate on homocysteine levels and with the reported correction of mild hyperhomocysteinemia by folic acid in individuals

- 37 -

with premature vascular disease, and in individuals with thermolabile MTHFR.

Although our cDNA is not long enough to encode the larger MTHFR polypeptide, it is capable of direct-
5 ing synthesis of the smaller isozyme. The ATG start codon for this polypeptide is within a good consensus sequence for translation initiation. Whether the isozyme is restricted to liver and what its role is in this tissue remain to be determined.

10 These data have identified a common genetic change in MTHFR which results in thermolability; our experiments do not directly address the relationship between this change and vascular disease. Nonetheless, this polymorphism represents a diagnostic test
15 for evaluation of MTHFR thermolability in hyperhomocysteinemia. Large case-control studies are required to evaluate the frequency of this genetic change in various forms of occlusive arterial disease and to examine the interaction between this genetic marker
20 and dietary factors. Well-defined populations need to be examined, since the limited data set thus far suggests that population-specific allele frequencies may exist. More importantly, however, the identification of a candidate genetic risk factor for vascular dis-
25 ease, which may be influenced by nutrient intake, represents a critical step in the design of appropriate therapies for the homocysteinemic form of arteriosclerosis.

30 cDNA FOR MTHFR AND ITS POTENTIAL UTILITY

A human cDNA for MTHFR (2.2 kb) has been isolated, as reported by us in Goyette et al. (*Nature Genetics*, 1994, 7:195-200) and Frosst et al. (*Nature Genetics*, 1995, 10:111-113). The cDNA has been
35 expressed *in vitro* to yield a MTHFR protein of

- 38 -

approximately 70 kDa (Frosst P et al., *Nature Genetics*, 1995, 10:111-113).

Using the cDNA sequence, mutations in patients with severe and mild MTHFR deficiency (Goyette P et al., *Nature Genetics*, 1994, 7:195-200; Goyette P et al., *Am. J. Hum. Genet.*, 1995, 56:1052-1059; Frosst P et al., *Nature Genetics*, 1995, 10:111-113) were identified.

The cDNA sequence is a necessary starting point for the detection of MTHFR sequence changes that would identify individuals at risk for cardiovascular and neurological diseases, as well as other disorders affected by folic acid metabolism. Diagnostic tests by DNA analysis are more efficient and accurate than testing by enzymatic/biochemical assays. Less blood is required and results are available in a shorter period of time. The tests could be performed as a routine operation in any laboratory that performs molecular genetic diagnosis, without the specialized reagents/expertise that is required for an enzyme-based test.

The second major utility of the cDNA would be in the design of therapeutic protocols, for correction of MTHFR deficiency. These protocols could directly involve the gene, as in gene therapy trials or in the use of reagents that could modify gene expression. Alternatively, the therapy might require knowledge of the amino acid sequence (derived from the cDNA sequence), as in the use of reagents that would modify enzyme activity. The identification of sequences and/or sequence changes in specific regions of the cDNA or protein, such as FAD binding sites or folate-binding sites, are useful in designing therapeutic protocols involving the above nutrients.

UTILITY OF INVENTION IN CLINICAL AND DIAGNOSTIC STUDIES

Coronary artery disease patients in Montreal (n=153) were studied to examine the frequency of the alanine to valine substitution. Fourteen percent of the patients were homozygous for this mutation. An analysis of 70 control individuals (free of cardiovascular disease) demonstrated that only seven % of these individuals were homozygous for the alanine to valine mutation.

Analysis of homocysteine levels in 123 men of the above patient group indicated that the mutant allele significantly raised homocysteine levels from 10.2 micromoles/L in homozygous normal men to 11.5 and 12.7 in heterozygotes and homozygous mutants, respectively.

Families with a child with spina bifida, a neural tube defect, have been examined for the presence of the alanine to valine mutation. Approximately 16% of mothers who had a child with spina bifida were homozygous for this mutation, while only 5% of control individuals were homozygous. Fathers of children with spina bifida also had an increased prevalence of the homozygous mutant genotype (10%) as did the affected children themselves (13%).

Table 4 indicates the interactive effect of folic acid with the homozygous mutant alanine to valine change. In a study of families from Framingham, Massachusetts and Utah, individuals who were homozygous mutant but had folate levels above 5 ng/ml did not have increased homocysteine levels compared to individuals with the normal or heterozygous genotype. However, individuals who were homozygous mutant but had folate levels below 5 ng/ml had homocysteine levels that were significantly higher than the other genotypes.

Table 4
Mean fasting and PML homocysteine levels for different
MTHFR genotypes

| Plasma Homocysteine | MTHFR genotype | | | P _{trend} |
|----------------------|----------------|---------------------|------------------|--------------------|
| | Normals (-/-) | Heterozygotes (+/-) | Homozygotes (++) | |
| N | 58 | 61 | 30 | |
| Fasting* | 9.4 | 9.2 | 12.1 | 0.02 |
| Folate <5 ng/mL | 10.2 | 10.4 | 15.2 | 0.002 |
| Folate ≥ 5ng/mL | 8.2 | 7.5 | 7.5 | 0.52 |
| Post-Methionine load | 30.0 | 30.9 | 31.3 | 0.62 |

5 * Significant interaction between folate levels and genotype (p=0.03)

Example III provides preliminary data for therapeutic intervention by folic acid supplementation to individuals who are homozygous for the alanine to valine change. The data suggest that higher levels of plasma folate would lead to normalization of homocysteine levels in mutant individuals and might prevent the occurrence of disorders associated with high homocysteine levels, such as cardiovascular disease, neural tube defects, and possibly other disorders. Folic acid supplementation for mutant individuals might also restore methionine and S-adenosylmethionine levels to normal. This would be relevant for disorders that are influenced by methylation, such as neoplasias, developmental anomalies, neurologic disease, etc.

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the

art to which the invention pertains and as may be applied to the essential features hereinbefore set forth, and as follows in the scope of the appended claims.

WE CLAIM:

1. A cDNA probe for human methylenetetrahydrofolate reductase (MTHFR).
2. Use of the probe of claim 1 for the identification of sequence abnormalities in patients with severe or mild MTHFR deficiency.
3. The use of claim 2, wherein said deficiency is selected from the group consisting of cardiovascular and neurological disorders and disorders influenced by folic acid metabolism.
4. Use of the probe of claim 1 for gene therapy to produce the MTHFR protein.
5. A human MTHFR protein which is encoded by the probe of claim 1.
6. Use of the probe of claim 1 and the protein of claim 4 for therapy of MTHFR-deficiency patients by dietary, biochemical or pharmacological approaches.

10 20 1/18 30 40 50
 * * * * *
 AAT TCC GGA GCC ATG GTG AAC GAA GCC AGA GGA AAC AGE AGE CTC AAC CCC
 TTA AGG CCT CGG TAC CAC TTG CTT CGG TCT CCT TTG TCG TCG GAG TTG GGG
 Asn Ser Gly Ala Met Val Asn Glu Ala Arg Gly Asn Ser Ser Leu Asn Pro>

60 70 80 90 100
 * * * * *
 TGC TTG GAG GGC AGT GCC AGC AGT GGC AGT GAG AGC TCC AAA GAT AGT TCG
 ACG AAC CTC CCG TCA CCG TCG TCA CCG TCA CTC TCG AGG TTT CTA TCA AGC
 Cys Leu Glu Gly Ser Ala Ser Ser Gly Ser Glu Ser Ser Lys Asp Ser Ser>

110 120 130 140 150
 * * * * *
 AGA TGT TCC ACC CCG GGC CTG GAC CCT GAG CCG CAT GAG AGA CTC CCG GAG
 TCT ACA AGG TGG GGC CCG GAC CTG GGA CTC GCC GTA CTC TCT GAG GCC CTC
 Arg Cys Ser Thr Pro Gly Leu Asp Pro Glu Arg His Glu Arg Leu Arg Glu>

160 170 180 190 200
 * * * * *
 AAG ATG AGG CCG CGA TTG GAA TCT GGT GAC AAG TGG TTC TCC CTG GAA TTC
 TTC TAC TCC GCC GGT AAC CTT AGA CCA CTG TTC ACC AAG AGG GAC CTT AAG
 Lys Met Arg Arg Arg Leu Glu Ser Gly Asp Lys Trp Phe Ser Leu Glu Phe>

210 220 230 240 250
 * * * * *
 TTC CCT CCT CGA ACT GGT GAG GGA GGT GTC AAT CTC ATC TCA AGG TTT GAC
 AAG GGA GGA GGT TGA CGA CTC CCT CGA CAG TTA GAG TAG AGT TCC AAA CTG
 Phe Pro Pro Arg Thr Ala Glu Gly Ala Val Asn Leu Ile Ser Arg Phe Asp>

260 270 280 290 300
 * * * * *
 CCG ATG GCA GCA GGT GGC CCC CTC TAC ATA GAC GTG ACC TGG CAC CCA GCA
 GCC TAC CGT CGT CCA CCG GGG GAG ATG TAT CTG CAC TGG ACC GTG GGT CGT
 Arg Met Ala Ala Gly Gly Pro Leu Tyr Ile Asp Val Thr Trp His Pro Ala>

310 320 330 340 350
 * * * * *
 GGT GAC CCT GGC TCA GAC AAG GAG ACC TCC TCC ATG ATG ATC GCC AGC ACC
 CCA CTG GGA CCG AGT CTG TTC CTC TGG AGG AGG TAC TAC TAG CCG TCG TGG
 Gly Asp Pro Gly Ser Asp Lys Glu Thr Ser Ser Met Met Ile Ala Ser Thr>

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2/18

360 370 380 390 400
 * * * * * * * *
 GCC GTG AAC TAC TGT GGC CTG GAG ACC ATC CTG CAC ATG ACC TGC TGC CGT
 CGG CAC TTG ATG ACA CCG GAC CTC TGG TAG GAC GTG TAC TGG ACG ACG GCA
 Ala Val Asn Tyr Cys Gly Leu Glu Thr Ile Leu His Met Thr Cys Cys Arg>

410 420 430 440 450
 * * * * * * * *
 CAG CGC CTG GAG GAG ATC ACG GGC CAT CTG CAC AAA GGT AAG CAG CTG GGC
 GTC GCG GAC CTC CTC TAG TGC CCG GTA GAC GTG TTT CGA TTC GTC GAC CCG
 Gln Arg Leu Glu Glu Ile Thr Gly His Leu His Lys Ala Lys Gln Leu Gly>

460 470 480 490 500 510
 * * * * * * * *
 CTG AAG AAC ATC ATG GCG CTG CCG GGA GAC CCA ATA GGT GAC CAG TGG GAA
 GAC TTC TTG TAG TAC CCG GAC GCC CCT CTG GGT TAT CCA CTG GTC ACC CTT
 Leu Lys Asn Ile Met Ala Leu Arg Gly Asp Pro Ile Gly Asp Gln Trp Glu>

 520 530 540 550 560
 * * * * * * * *
 GAG GAG GAG GGA GGC TTC AAC TAC GCA GTG GAC CTG GTG AAG CAC ATC CGA
 CTC CTC CTC CCT CCG AAG TTG ATG CGT CAC CTG GAC CAC TTC GTG TAG GGT
 Glu Glu Glu Gly Gly Phe Asn Tyr Ala Val Asp Leu Val Lys His Ile Arg>

 570 580 590 600 610
 * * * * * * * *
 AGT GAG TTT GGT GAC TAC TTT GAC ATC TGT GTG GCA GGT TAC CCC AAA GGC
 TCA CTC AAA CCA CTG ATG AAA CTG TAG ACA CAC CGT CCA ATG GGG TTT CCG
 Ser Glu Phe Gly Asp Tyr Phe Asp Ile Cys Val Ala Gly Tyr Pro Lys Gly>

 620 630 640 650 660
 * * * * * * * *
 CAC CCC GAA GCA GGG AGC TTT GAG GGT GAC CTG AAG CAC TTG AAG GAG AAG
 GTG GGG CTT CGT CCC TCG AAA CTC CGA CTG GAC TTC GTG AAC TTC CTC TTC
 His Pro Glu Ala Gly Ser Phe Glu Ala Asp Leu Lys His Leu Lys Glu Lys>

 670 680 690 700 710
 * * * * * * * *
 GTG TCT GCG GGA GCC GAT TTC ATC ATC ACG CAG CTT TTC TTT GAG GGT GAC
 CAC AGA CGC CCT CCG CTA AAG TAG TAG TGC GTC GAA AAG AAA CTC CGA CTG
 Val Ser Ala Gly Ala Asp Phe Ile Ile Thr Gln Leu Phe Phe Glu Ala Asp>

18

3/18

720 730 740 750 760
 * * * * *
 ACA TTC TTC CGC TTT GTG AAG GCA TGC ACC GAC ATG GGC ATC ACT TGC CCC
 TGT AAG AAG GCG AAA CAC TTC CGT ACG TGG CTG TAC CCG TAG TGA ACG GGG
 Thr Phe Phe Arg Phe Val Lys Ala Cys Thr Asp Met Gly Ile Thr Lys Pro>

770 780 790 800 810
 * * * * *
 ATC GTC CCC GGG ATC TTT CCC ATC CAG GGC TAC CAC TCC CTT CCG CAG CTT
 TAG CAG GGG CCC TAG AAA GGG TAG GTC CCG ATG GTG AGG GAA GCC GTC GAA
 Ile Val Pro Gly Ile Phe Pro Ile Gln Gly Tyr His Ser Leu Arg Gln Leu>

820 830 840 850 860
 * * * * *
 GTG AAG CTG TCC AAG CTG GAG GTG CCA CAG GAG ATC AAG GAC GTG ATT GAG
 CAC TTC GAC AGG TTC GAC CTC CAC GGT GTC CTC TAG TTC CTG CAC TAA CTC
 Val Lys Leu Ser Lys Leu Glu Val Pro Gln Glu Ile Lys Asp Val Ile Glu>

870 880 890 900 910
 * * * * *
 CCA ATC AAA GAC AAC GAT GCT GCC ATC CGC AAC TAT GGC ATC GAG CTG GCC
 GGT TAG TTT CTG TTG CTA CGA CCG TAG GCG TTG ATA CCG TAG CTC GAC CCG
 Pro Ile Lys Asp Asn Asp Ala Ala Ile Arg Asn Tyr Gly Ile Glu Leu Ala>

920 930 940 950 960
 * * * * *
 GTG AGC CTG TGC CAG GAG CTT CTG GCC AGT GGC TTG GTG CCA GGC CTC CAC
 CAC TCG GAC ACG GTC CTC GAA GAC CCG TCA CCG AAC CAC GGT CCG GAG GTG
 Val Ser Leu Cys Gln Glu Leu Leu Ala Ser Gly Leu Val Pro Gly Leu His>

970 980 990 1000 1010 1020
 * * * * *
 TTC TAC ACC CTC AAC CGC GAG ATG GCT ACC ACA GAG GTG CTG AAG CGC CTG
 AAG ATG TGG GAG TTG GCG CTC TAC CGA TGG TGT CTC CAC GAC TTC GCG GAC
 Phe Tyr Thr Leu Asn Arg Glu Met Ala Thr Thr Glu Val Leu Lys Arg Leu>

1030 1040 1050 1060 1070
 * * * * *
 GGG ATG TGG ACT GAG GAC CCC AGG CGT CCC CTA CCC TGG GCT CTC AGT GCC
 CCC TAC ACC TGA CTC CTG GGG TCC GCA GGG GAT GGG ACC CGA GAG TCA CCG
 Gly Met Trp Thr Glu Asp Pro Arg Arg Pro Leu Pro Trp Ala Leu Ser Ala>

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4/18

| | | | | |
|---|------|------|------|------|
| 1080 | 1090 | 1100 | 1110 | 1120 |
| x | x | x | x | x |
| CAC CCC AAG CGC CGA GAG GAA GAT GTA CGT CCC ATC TTC TGG GCC TCC AGA | | | | |
| GTG GGG TTC GCG GCT CTC CTT CTA CAT GCA GGG TAG AAG ACC CGG AGG TCT | | | | |
| His Pro Lys Arg Arg Glu Glu Asp Val Arg Pro Ile Phe Trp Ala Ser Arg | | | | |

| | | | | |
|---|------|------|------|------|
| 1130 | 1140 | 1150 | 1160 | 1170 |
| x | x | x | x | x |
| CCA AAG AGT TAC ATC TAC CGT ACC CAG GAG TGG GAC GAG TTC CTT AAC GGC | | | | |
| GGT TTC TCA ATG TAG ATG GCA TGG GTC CTC ACC CTG CTC AAG GGA TTG CCG | | | | |
| Pro Lys Ser Tyr Ile Tyr Arg Thr Gln Glu Trp Asp Glu Phe Pro Asn Gly | | | | |

| | | | | |
|---|------|------|------|------|
| 1180 | 1190 | 1200 | 1210 | 1220 |
| x | x | x | x | x |
| CGC TGG GGC AAT TCC TCT TCC CCT GCC TTT GGG GAG CTG AAG GAC TAC TAC | | | | |
| GCG ACC CCG TTA AGG AGA AGG GGA CCG AAA CCC CTC GAC TTC CTG ATG ATG | | | | |
| Arg Trp Gly Asn Ser Ser Ser Pro Ala Phe Gly Glu Leu Lys Asp Tyr Tyr | | | | |

| | | | | |
|---|------|------|------|------|
| 1230 | 1240 | 1250 | 1260 | 1270 |
| x | x | x | x | x |
| CTC TTC TAC CTG AAG AGC AAG TCC CCC AAG GAG GAG CTG CTG AAG ATG TGG | | | | |
| GAG AAG ATG GAC TTC TCG TTC AGG GGG TTC CTC CTC GAC GAC TTC TAC ACC | | | | |
| Leu Phe Tyr Leu Lys Ser Lys Ser Pro Lys Glu Glu Leu Leu Lys Met Trp | | | | |

| | | | | |
|---|------|------|------|------|
| 1280 | 1290 | 1300 | 1310 | 1320 |
| x | x | x | x | x |
| GGG GAG GAG CTG ACC AGT GAA GCA AGT GTC TTT GAA GTC TTT GTT CTT TAC | | | | |
| CCC CTC CTC GAC TGG TCA CTT CGT TCA CAG AAA CTT CAG AAA CAA GAA ATG | | | | |
| Gly Glu Glu Leu Thr Ser Glu Ala Ser Val Phe Glu Val Phe Val Leu Tyr | | | | |

| | | | | |
|---|------|------|------|------|
| 1330 | 1340 | 1350 | 1360 | 1370 |
| x | x | x | x | x |
| CTC TCG GGA GAA CCA AAC CGG AAT GGT CAC AAA GTG AET TGC CTG CCC TGG | | | | |
| GAG AGC CCT CTT GGT TTG GCC TTA CCA GTG TTT CAC TGA ACG GAC GGG ACC | | | | |
| Leu Ser Gly Glu Pro Asn Arg Asn Gly His Lys Val Thr Cys Leu Pro Trp | | | | |

| | | | | |
|---|------|------|------|------|
| 1380 | 1390 | 1400 | 1410 | 1420 |
| x | x | x | x | x |
| AAC GAT GAG CCC CTG GCG GGT GAG ACC AGC CTG CTG AAG GAG GAG CTG CTG | | | | |
| TTG CTA CTC GGG GAC CGC CGA CTC TGG TCG GAC GAC TTC CTC CTC GAC GAC | | | | |
| Asn Asp Glu Pro Leu Ala Ala Glu Thr Ser Leu Leu Lys Glu Glu Leu | | | | |

FIG. 11

5/18

1430 1440 1450 1460 1470

x x x x x x x

CGG GTG AAC CGC CAG GGC ATC CTC ACC ATC AAC TCA CAG CCC AAC ATC AAC

CCC CAC TTG GCG GTC CCG TAG GAG TGG TAG TTG AGT GTC GGG TTG TAG TTG

Arg Val Asn Arg Gln Gly Ile Leu Thr Ile Asn Ser Gln Pro Asn Ile Asn>

1480 1490 1500 1510 1520 1530

x x x x x x x

GGG AAG CCG TCC TCC GAC CCC ATC GTG GGC TGG GGC CCC AGC GGG GGC TAT

CCC TTC GGC AGG AGG CTG GGG TAG CAC CCG ACC CCG GGG TCG CCC CCG ATA

Gly Lys Pro Ser Ser Asp-Pro Ile Val Gly Trp Gly Pro Ser Gly Gly Tyr>

1540 1550 1560 1570 1580

x x x x x x x

GTC TTC CAG AAG GCC TAC TTA GAG TTT TTC ACT TCC CGC GAG ACA GCG GAA

CAG AAG GTC TTC CCG ATG AAT CTC AAA AAG TGA AGG GCG CTC TGT CGC CTT

Val Phe Gln Lys Ala Tyr Leu Glu Phe Phe Thr Ser Arg Glu Thr Ala Glu>

1590 1600 1610 1620 1630

x x x x x x x

GCA CTT CTG CAA GTG CTG AAG AAG TAC GAG CTC CCG GTT AAT TAC CAC CTT

CGT GAA GAC GTT CAC GAC TTC TTC ATG CTC GAG GCC CAA TTA ATG GTG GAA

Ala Leu Leu Gln Val Leu Lys Lys Tyr Glu Leu Arg Val Asn Tyr His Leu>

1640 1650 1660 1670 1680

x x x x x x x

GTC AAT GTG AAG GGT GAA AAC ATC ACC AAT GCC CCT GAA CTG CAG CCG AAT

CAG TTA CAC TTC CCA CTT TTG TAG TGG TTA CCG GGA CTT GAC GTC GGC TTA

Val Asn Val Lys Gly Glu Asn Ile Thr Asn Ala Pro Glu Leu Gln Pro Asn>

1690 1700 1710 1720 1730

x x x x x x x

GCT GTC ACT TGG GGC ATC TTC CCT GGG CGA GAG ATC ATC CAG CCC ACC GTA

CGA CAG TGA ACC CCG TAG AAG GGA CCC GGT CTC TAG TAG GTC GGG TGG CAT

Ala Val Thr Trp Gly Ile Phe Pro Gly Arg Glu Ile Ile Gln Pro Thr Val>

1740 1750 1760 1770 1780

x x x x x x x

GTG GAT CCC GTC AGC TTC ATG TTC TGG AAG GAC GAG GCC TTT GCC CTG TGG

CAC CTA GGG CAG TCG AAG TAC AAG ACC TTC CTG CTC CCG AAA CCG GAC ACC

Val Asp Pro Val Ser Phe Met Phe Trp Lys Asp Glu Ala Phe Ala Leu Trp>

FIG. 1E

6/18

1790 1800 1810 1820 1830
 * * * * *
 ATT GAG CGG TGG GGA AAG CTG TAT GAG GAG GAG TCC CCG TCC CGC ACC ATC
 TAA CTC GCC ACC CCT TTC GAC ATA CTC CTC CTC AGG GGC AGG GCG TGG TAG
 Ile Glu Arg Trp Gly Lys Leu Tyr Glu Glu Glu Ser Pro Ser Arg Thr Ile>

1840 1850 1860 1870 1880
 * * * * *
 ATC CAG TAC ATC CAC GAC AAC TAC TTC CTG GTC AAC CTG GTG GAC AAT GAC
 TAG GTC ATG TAG GTG CTG TTG ATG AAG GAC CAG TTG GAC CAC CTG TTA CTG
 Ile Gln Tyr Ile His Asp Asn Tyr Phe Leu Val Asn Leu Val Asp Asn Asp>

1890 1900 1910 1920 1930
 * * * * *
 TTC CCA CTG GAC AAC TGC CTC TGG CAG GTG GTG GAA GAC ACA TTG GAG CTT
 AAG GGT GAC CTG TTG ACG GAG ACC GTC CAC CAC CTT CTG TGT AAC CTC GAA
 Phe Pro Leu Asp Asn Cys Leu Trp Gln Val Val Glu Asp Thr Leu Glu Leu>

1940 1950 1960 1970 1980 1990
 * * * * * *
 CTC AAC AGG CCC ACC CAG AAT GCG AGA GAA ACG GAG GET CCA TGACCTGCG
 GAG TTG TCC GGG TGG GTC TTA CGC TCT CTT TGC CTC CGA GGT ACTGGGACGC
 Leu Asn Arg Pro Thr Gln Asn Ala Arg Glu Thr Glu Ala Pro>

2000 2010 2020 2030 2040 2050
 * * * * * *
 TCCGACGCC CTGCTTGGG GCACTCTG TCCCGCTTC CTCCTCCACA GTGCTGCTTC
 AGGACTGCGG GACGCAACT CCGTGAGGAC AGGGCGGAAG GAGGAGGTGT CACGACGAAG

2060 2070 2080 2090 2100 2110
 * * * * * *
 TCTTGGGAAC TCCACTCTCC TTCGTGTCTC TCCACCCCG GCCTCCACTC CCCCACCTGA
 AGAACCTTGG AGGTGAGAGG AAGCACAGAG AGGGTGGGGC CCGAGGTGAG GGGGTGGACT

2120 2130 2140 2150 2160 2170
 * * * * * *
 CAATGGCAGC TAGACTGGAG TGAGGCTTCC AGGCTCTTCC TGGACCTGAG TCGGCCCCAC
 GTTACCTGTC ATCTGACCTC ACTCCGAAGG TCCGAGAAGG ACCTGGACTC AGCCGGGGTG

2180 2190 2200 2210 2220
 * * * * *
 ATGGGAACCT AGTACTCTCT GCTCTAAAAA AAAAAAAAAA AAAGGAATTC
 TACCTTTGGA TCATGAGAGA CGAGATTTT TTTTTTTTTT TTTCTTAAAG

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7/18

AMVNE ARGNS SLNPC LEGSA SSGSE SSKDS SRCST PGLDP ^{ms}ERHER LREKM RRRLE S--GDKW ESLEF ^{ms}ffHas qRdal nqsla evqGqin VSFEF ^{ms}ffHan qREal nqsla evqGqin VSFEF ^{ms}iRdLy haraspf ISLEF
 mthfr
 ecometf
 stymetf
 ysRADI

FPPRT AEGAV NLISR EDRMA AGGPL YIDVT WHPAG DPGSD KETSS MMIAS TAVNY CGLET ILHMT
 FPPRT sEmeq tLwns iDRIs slKPK fvsVT y--ga nsger drThs i-lkg ik-dr tGLEa aphIT
 FPPRT sEmeq tLwns iDRIs slKPK fvsVT y--ga nsger drThs v-lkg ik-er tGLEa aphIT
 FPPkT eLGtr NLmeR mHRmt AldPL fItVT W--ga -gGtt aEktI t-lAS lAqqt lniPv cmHIT
 mthfr
 ecometf
 stymetf
 ysRADI

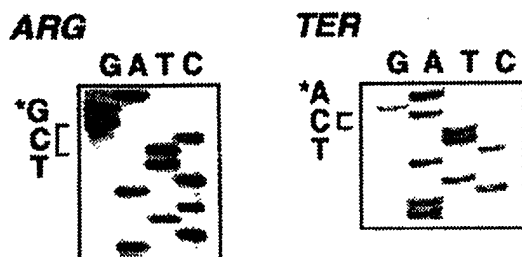
CCRQR LEEIT GHLHK AKQLG LKNIM ALRGD ^{*}-PIGDQ WEEEE GGFNY AVGLV KHIRS ^{*}EFGDY FDICV
 Cidat pdElr tiard ywnng irhIv ALRGD lPpGsg kPE-- ---mY AsdLV tllk- EvaD- FDIsV
 Cidat rdElr tiard ywnng irhIv ALRGD lPpGsg kPE-- ---mY AadLV gllk- EvaD- FDIsV
 Cntte kaiId daLdr cynag irNI l ALRGn lPIGvv WlvSq snrll nmrLf>
 mthfr
 ecometf
 stymetf
 ysRADI

AGYPK GHPEA GSFEA DLKHL KEKVS AGADE IITQL FFEAD TFFRF VKACT DMGIT CPIVP GIFPI
 AaYPE VHPEA KSaqa DLinL KrKVd AGAnr aITQf FFdve sylRF rdrCv saGId veliP GIlPv
 AaYPE VHPEA KSaqa DLinL KrKVd AGAnr aITQf FFdve sylRF rdrCv saGId veliP GIlPv
 mthfr
 ecometf
 stymetf

QGYHS LRQLV KLSKL EVPQE IKDVI EPIKD NDAAI RN-YGI ELAVS LCOEL LASGL VPGLH FYTLN
 snfkq akkfa dmtnv riPaw maqmf dgl-D dDAet RklvGa niAmd mvkil sreG- VkdFH FYTLN
 snfkq akkfa dmtnv riPsw mslmf Egl-D nDAet RklvGa niAmd mvkil sreG- VkdFH FYTLN
 mthfr
 ecometf
 stymetf

R-EMAT TEVLK RLGMW TEDPR RPLPW ALSAH PKRRE EDVRP IFWAS RPKSY IYRID EWDEE PNGRW
 RaEMsy a-ich tLGvr pgi>
 RaEMsy a-ich tLGvr pgi>
 400.
 GNSSS PAFGE LKDYI LFYLK SKSPK E mthfr

8/18



FI 3A



FI 3B

SUBSTITUTE SHEET

9/18

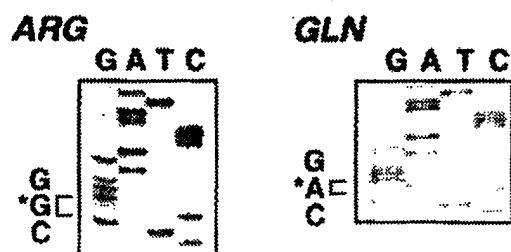


Fig. 4A

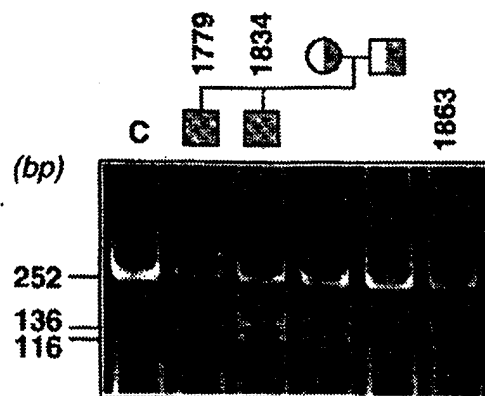
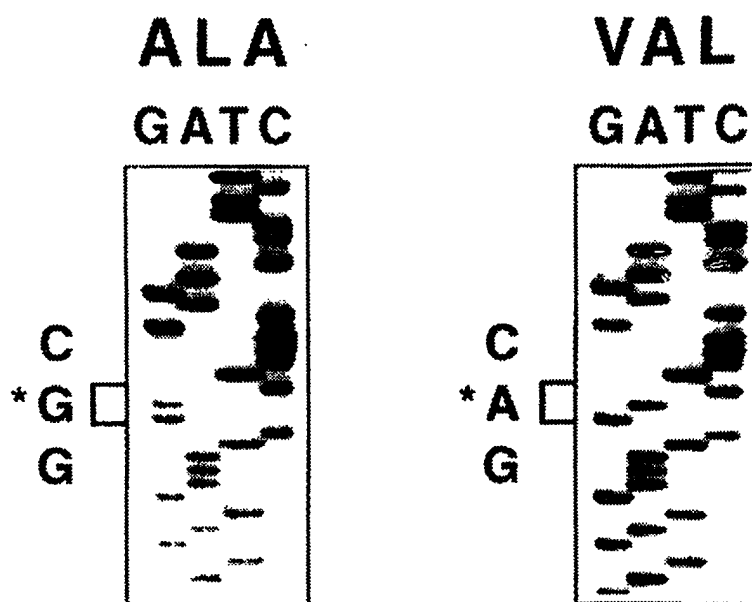


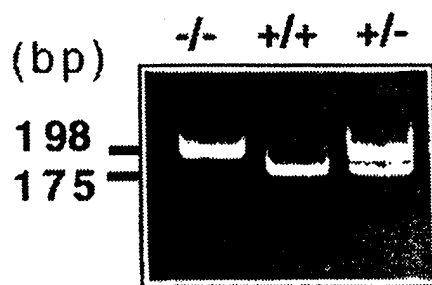
Fig. 4B

SUBSTITUTE SHEET

10/18



FISSA



FISSB

SUBSTITUTE SHEET

— 11/18

| | | | | | | | | | | | | | | | | | | | | |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| AAT | TCC | GGA | GCC | ATG | GTG | AAC | GAA | GCC | AGA | GGA | AAC | AGC | AGC | CTC | AAC | CCC | TGC | TTG | GAG | 60 |
| | | | | Met | Val | Asn | Glu | Ala | Arg | Gly | Asn | Ser | Ser | Leu | Asn | Pro | Cys | Leu | Glu | 16 |
| GGC | AGT | GCC | AGC | AGT | GGC | AGT | GAG | AGC | TCC | AAA | GAT | AGT | TGC | AGA | TGT | TCC | ACC | CCG | GGC | 120 |
| Gly | Ser | Ala | Ser | Ser | Gly | Ser | Glu | Ser | Ser | Lys | Asp | Ser | Ser | Arg | Cys | Ser | Thr | Pro | Gly | 36 |
| CTG | GAC | CCT | GAG | CGG | CAT | GAG | AGA | CTC | CGG | GAG | AAG | ATG | AGG | CGG | CGA | TTG | GAA | TCT | GGT | 180 |
| Leu | Asp | Pro | Glu | Arg | His | Glu | Arg | Leu | Arg | Glu | Lys | Met | Arg | Arg | Arg | Leu | Glu | Ser | Gly | 56 |
| GAC | AAG | TGG | TTC | TCC | CTG | GAA | TTC | TTC | CCT | CCT | CGA | ACT | GCT | GAG | GGA | GCT | GTC | AAT | CTC | 240 |
| Asp | Lys | Trp | Phe | Ser | Leu | Glu | Phe | Phe | Pro | Pro | Arg | Thr | Ala | Glu | Gly | Ala | Val | Asn | Leu | 76 |
| ATC | TCA | AGG | TTT | GAC | CGG | ATG | GCA | GCA | GGT | GGC | CCC | CTC | TAC | ATA | GAC | GTG | ACC | TGG | CAC | 300 |
| Ile | Ser | Arg | Phe | Asp | Arg | Met | Ala | Ala | Gly | Gly | Pro | Leu | Tyr | Ile | Asp | Val | Thr | Trp | His | 96 |
| CCA | GCA | GGT | GAC | CCT | GGC | TCA | GAC | AAG | GAG | ACC | TCC | TCC | ATG | ATG | ATC | GCC | AGC | ACC | GCC | 360 |
| Pro | Ala | Gly | Asp | Pro | Gly | Ser | Asp | Lys | Glu | Thr | Ser | Ser | Met | Met | Ile | Ala | Ser | Thr | Ala | 116 |
| GTG | AAC | TAC | TGT | GGC | CTG | GAG | ACC | ATC | CTG | CAC | ATG | ACC | TGC | TGC | CGT | CAG | CGC | CTG | GAG | 420 |
| Val | Asn | Tyr | Cys | Gly | Leu | Glu | Thr | Ile | Leu | His | Met | Thr | Cys | Cys | Arg | Gln | Arg | Leu | Glu | 136 |
| GAG | ATC | ACG | GGC | CAT | CTG | CAC | AAA | GCT | AAG | CAG | CTG | GGC | CTG | AAG | AAC | ATC | ATG | GCG | CTG | 480 |
| Glu | Ile | Thr | Gly | His | Leu | His | Lys | Ala | Lys | Gln | Leu | Gly | Leu | Lys | Asn | Ile | Met | Ala | Leu | 156 |
| CGG | GGA | GAC | CCA | ATA | GGT | GAC | CAG | TGG | GAA | GAG | GAG | GAG | GGA | GGC | TTC | AAC | TAC | GCA | GTG | 540 |
| Arg | Gly | Asp | Pro | Ile | Gly | Asp | Gln | Trp | Glu | Glu | Glu | Glu | Gly | Gly | Phe | Asn | Tyr | Ala | Val | 176 |
| GAC | CTG | GTG | AAG | CAC | ATC | CGA | AGT | GAG | TTT | GGT | GAC | TAC | TTT | GAC | ATC | TGT | GTG | GCA | GGT | 600 |
| Asp | Leu | Val | Lys | His | Ile | Arg | Ser | Glu | Phe | Gly | Asp | Tyr | Phe | Asp | Ile | Cys | Val | Ala | Gly | 196 |
| TAC | CCC | AAA | GGC | CAC | CCC | GAA | GCA | GGG | AGC | TTT | GAG | GCT | GAC | CTG | AAG | CAC | TTG | AAG | GAG | 660 |
| Tyr | Pro | Lys | Gly | His | Pro | Glu | Ala | Gly | Ser | Phe | Glu | Ala | Asp | Leu | Lys | His | Leu | Lys | Glu | 216 |
| AAG | GTG | TCT | GCG | GGA | GCC | GAT | TTC | ATC | ATC | ACG | CAG | CTT | TTC | TTT | GAG | GCT | GAC | ACA | TTC | 720 |
| Lys | Val | Ser | Ala | Gly | Ala | Asp | Phe | Ile | Ile | Thr | Gln | Leu | Phe | Phe | Glu | Ala | Asp | Thr | Phe | 236 |

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12/18

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|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|------|
| TTC | CGC | TTT | GTG | AAG | GCA | TGC | ACC | GAC | ATG | GGC | ATC | AET | TGC | CCC | ATC | GTC | CCC | GGG | ATC | 780 |
| Phe | Arg | Phe | Val | Lys | Ala | Cys | Thr | Asp | Met | Gly | Ile | Thr | Lys | Pro | Ile | Val | Pro | Gly | Ile | 256 |
| TTT | CCC | ATC | CAG | GGC | TAC | CAC | TCC | CTT | GGG | CAG | CTT | GTG | AAG | CTG | TCC | AAG | CTG | GAG | GTG | 840 |
| Phe | Pro | Ile | Gln | Gly | Tyr | His | Ser | Leu | Arg | Gln | Leu | Val | Lys | Leu | Ser | Lys | Leu | Glu | Val | 276 |
| CCA | CAG | GAG | ATC | AAG | GAC | GTG | ATT | GAG | CCA | ATC | AAA | GAC | AAC | GAT | GCT | GCC | ATC | CGC | AAC | 900 |
| Pro | Gln | Glu | Ile | Lys | Asp | Val | Ile | Glu | Pro | Ile | Lys | Asp | Asn | Asp | Ala | Ala | Ile | Arg | Asn | 296 |
| TAT | GGC | ATC | GAG | CTG | GCC | GTG | AGC | CTG | TGC | CAG | GAG | CTT | CTG | GCC | AGT | GGC | TTG | GTG | CCA | 960 |
| Tyr | Gly | Ile | Glu | Leu | Ala | Val | Ser | Leu | Cys | Gln | Glu | Leu | Leu | Ala | Ser | Gly | Leu | Val | Pro | 316 |
| GGC | CTC | CAC | TTC | TAC | ACC | CTC | AAC | CGC | GAG | ATG | GCT | ACC | ACA | GAG | GTG | CTG | AAG | CGC | CTG | 1020 |
| Gly | Leu | His | Phe | Tyr | Thr | Leu | Asn | Arg | Glu | Met | Ala | Thr | Thr | Glu | Val | Leu | Lys | Arg | Leu | 336 |
| GGG | ATG | TGG | ACT | GAG | GAC | CCC | AGG | CGT | CCC | CTA | CCC | TGG | GCT | CTC | AGT | GCC | CAC | CCC | AAG | 1080 |
| Gly | Met | Trp | Thr | Glu | Asp | Pro | Arg | Arg | Pro | Leu | Pro | Trp | Ala | Leu | Ser | Ala | His | Pro | Lys | 356 |
| CGC | CGA | GAG | GAA | GAT | GTA | CGT | CCC | ATC | TTC | TGG | GCC | TCC | AGA | CCA | AAG | AGT | TAC | ATC | TAC | 1140 |
| Arg | Arg | Glu | Glu | Asp | Val | Arg | Pro | Ile | Phe | Trp | Ala | Ser | Arg | Pro | Lys | Ser | Tyr | Ile | Tyr | 376 |
| CGT | ACC | CAG | GAG | TGG | GAC | GAG | TTC | CTT | AAC | GGC | CGC | TGG | GGC | AAT | TCC | TCT | TCC | CTT | GCC | 1200 |
| Arg | Thr | Gln | Glu | Trp | Asp | Glu | Phe | Pro | Asn | Gly | Arg | Trp | Gly | Asn | Ser | Ser | Ser | Pro | Ala | 396 |
| TTT | GGG | GAG | CTG | AAG | GAC | TAC | TAC | CTC | TTC | TAC | CTG | AAG | AGC | AAG | TCC | CCC | AAG | GAG | GAG | 1260 |
| Phe | Gly | Glu | Leu | Lys | Asp | Tyr | Tyr | Leu | Phe | Tyr | Leu | Lys | Ser | Lys | Ser | Pro | Lys | Glu | Glu | 416 |
| CTG | CTG | AAG | ATG | TGG | GGG | GAG | GAG | CTG | ACC | AGT | GAA | GCA | AGT | GTC | TTT | GAA | GTC | TTT | GTT | 1320 |
| Leu | Leu | Lys | Met | Trp | Gly | Glu | Glu | Leu | Thr | Ser | Glu | Ala | Ser | Val | Phe | Glu | Val | Phe | Val | 436 |
| CTT | TAC | CTC | TGC | GGA | GAA | CCA | AAC | CGG | AAT | GGT | CAC | AAA | GTG | ACT | TGC | CTG | CCC | TGG | AAC | 1380 |
| Leu | Tyr | Leu | Ser | Gly | Glu | Pro | Asn | Arg | Asn | Gly | His | Lys | Val | Thr | Cys | Leu | Pro | Trp | Asn | 456 |
| GAT | GAG | CCC | CTG | GGG | GCT | GAG | ACC | AGC | CTG | CTG | AAG | GAG | GAG | CTG | CTG | CGG | GTG | AAC | CGC | 1440 |
| Asp | Glu | Pro | Leu | Ala | Ala | Glu | Thr | Ser | Leu | Leu | Lys | Glu | Glu | Leu | Leu | Arg | Val | Asn | Arg | 476 |

FIG. 6B

13/18

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      x           x           x           x           x           x
CAG GGC ATC CTC ACC ATC AAC TCA CAG CCC AAC ATC AAC GGG AAG CCG TCC TCC GAC CCC 1500
Gln Gly Ile Leu Thr Ile Asn Ser Gln Pro Asn Ile Asn Gly Lys Pro Ser Ser Asp Pro 496

      x           x           x           x           x           x
ATC GTG GGC TGG GGC CCC AGC GGG GGC TAT GTC TTC CAG AAG GCC TAC TTA GAG TTT TTC 1560
Ile Val Gly Trp Gly Pro Ser Gly Gly Tyr Val Phe Gln Lys Ala Tyr Leu Glu Phe Phe 516

      x           x           x           x           x           x
ACT TCC CGC GAG ACA GCG GAA GCA CTT CTG CAA GTG CTG AAG AAG TAC GAG CTC CGG GTT 1620
Thr Ser Arg Glu Thr Ala Glu Ala Leu Leu Gln Val Leu Lys Lys Tyr Glu Leu Arg Val 536

      x           x           x           x           x           x
AAT TAC CAC CTT GTC AAT GTG AAG GGT GAA AAC ATC ACC AAT GCC CTT GAA CTG CAG CCG 1680
Asn Tyr His Leu Val Asn Val Lys Gly Glu Asn Ile Thr Asn Ala Pro Glu Leu Gln Pro 556

      x           x           x           x           x           x
AAT GCT GTC ACT TGG GGC ATC TTC CTT GGG CGA GAG ATC ATC CAG CCC ACC GTA GTG GAT 1740
Asn Ala Val Thr Trp Gly Ile Phe Pro Gly Arg Glu Ile Ile Glu Pro Thr Val Val Asp 576

      x           x           x           x           x           x
CCC GTC AGC TTC ATG TTC TGG AAG GAC GAG GCC TTT GCC CTG TGG ATT GAG CCG TGG GGA 1800
Pro Val Ser Phe Met Phe Trp Lys Asp Glu Ala Phe Ala Leu Trp Ile Glu Arg Trp Gly 596

      x           x           x           x           x           x
AAG CTG TAT GAG GAG GAG TCC CCG TCC CGC ACC ATC ATC CAG TAC ATC CAC GAC AAC TAC 1860
Lys Leu Tyr Glu Glu Glu Ser Pro Ser Arg Thr Ile Ile Gln Tyr Ile His Asp Asn Tyr 616

      x           x           x           x           x           x
TTC CTG GTC AAC CTG GTG GAC AAT GAC TTC CCA CTG GAC AAC TGC CTC TGG CAG GTG GTG 1920
Phe Leu Val Asn Leu Val Asp Asn Asp Phe Pro Leu Asp Asn Cys Leu Trp Gln Val Val 636

      x           x           x           x           x           x
GAA GAC ACA TTG GAG CTT CTC AAC AGG CCC ACC CAG AAT GCG AGA GAA ACG GAG GCT CCA 1980
Glu Asp Thr Leu Glu Leu Leu Asn Arg Pro Thr Gln Asn Ala Arg Glu Thr Glu Ala Pro 656

      x           x           x           x           x           x
TGA CCC TGC GTC CTG ACG CCC TGC GTT GGA GCC ACT CCT GTC CCG CCT TCC TCC TCC ACA 2040
End

      x           x           x           x           x           x
GTG CTG CTT CTC TTG GGA AET CCA CTC TCC TTC GTG TCT CTC CCA CCC CGG CCT CCA CTC 2100

      x           x           x           x           x           x
CCC CAC CTG ACA ATG GCA GGT AGA CTG GAG TGA GGC TTC CAG GCT CTT CCT GGA CCT GAG 2160

      x           x           x           x           x           x
TEG GGC CCA CAT GGG AAC CTA GTA CTC TCT GCT CTA AAA AAA AAA AAA AAA AAG GAA TT 2220

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14/18

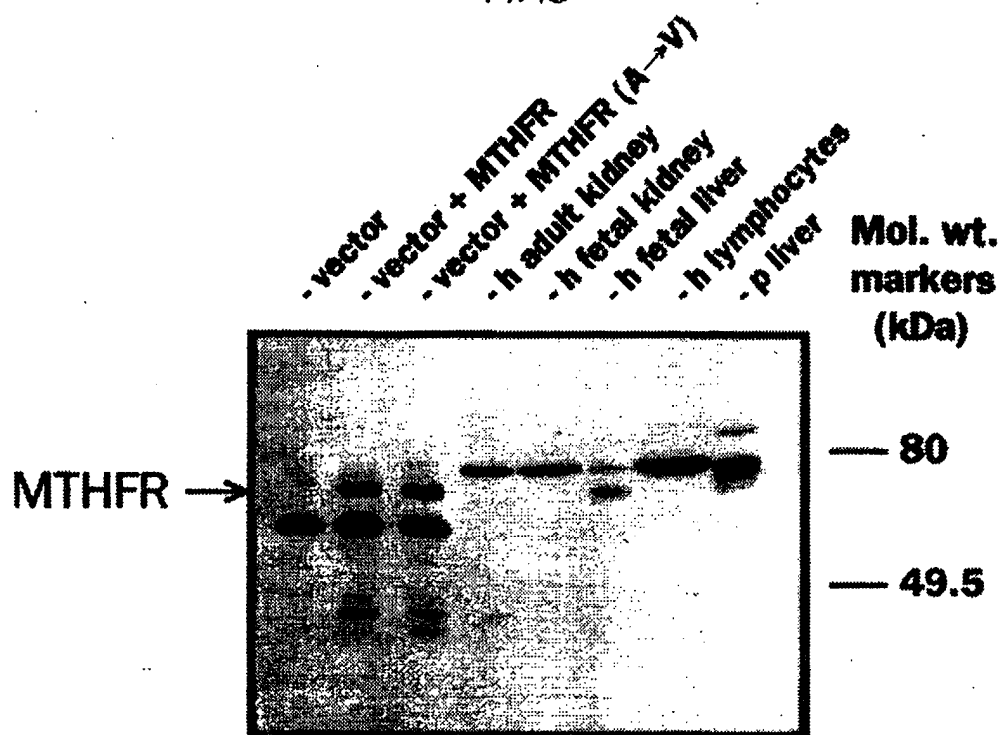


FIG. 7A

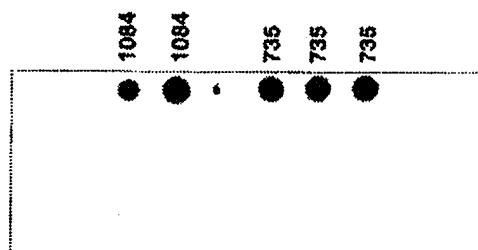


FIG. 10A

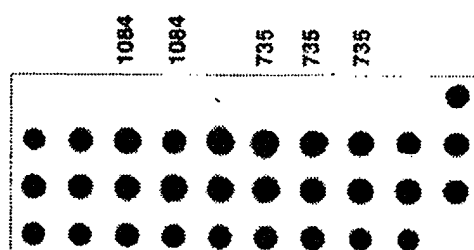


FIG. 10B

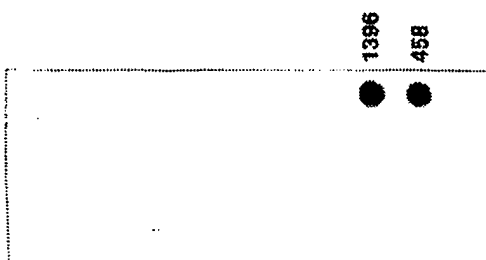


FIG. 10C

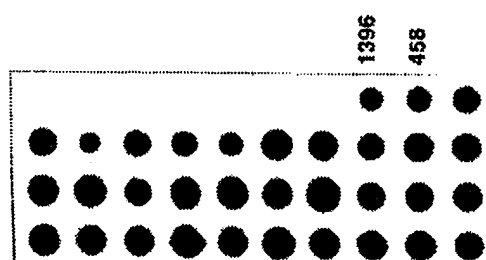
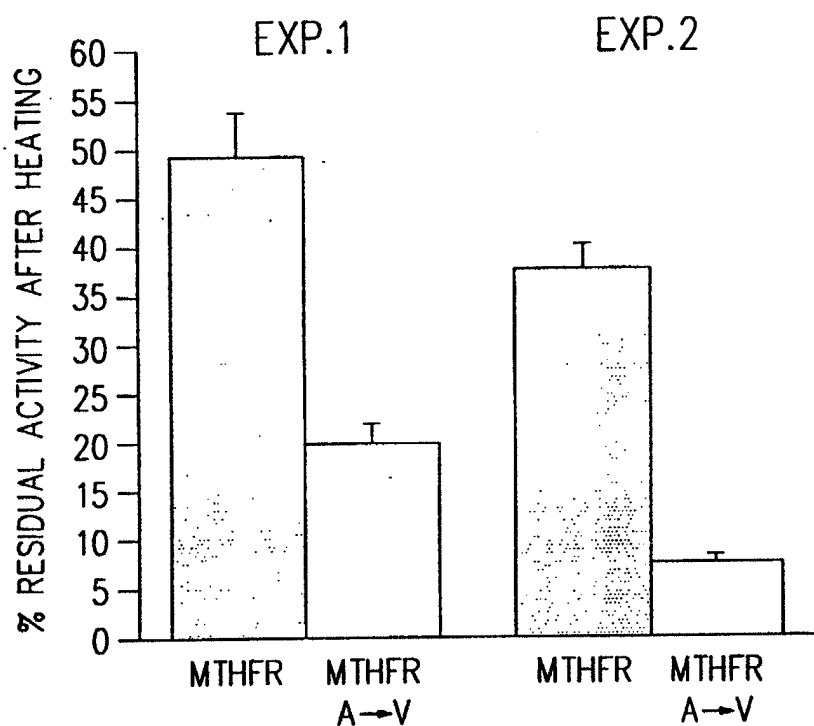


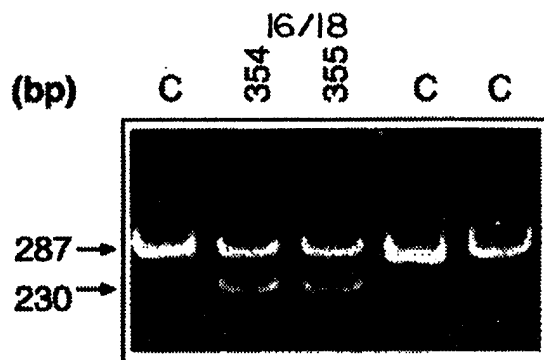
FIG. 10D

15/18

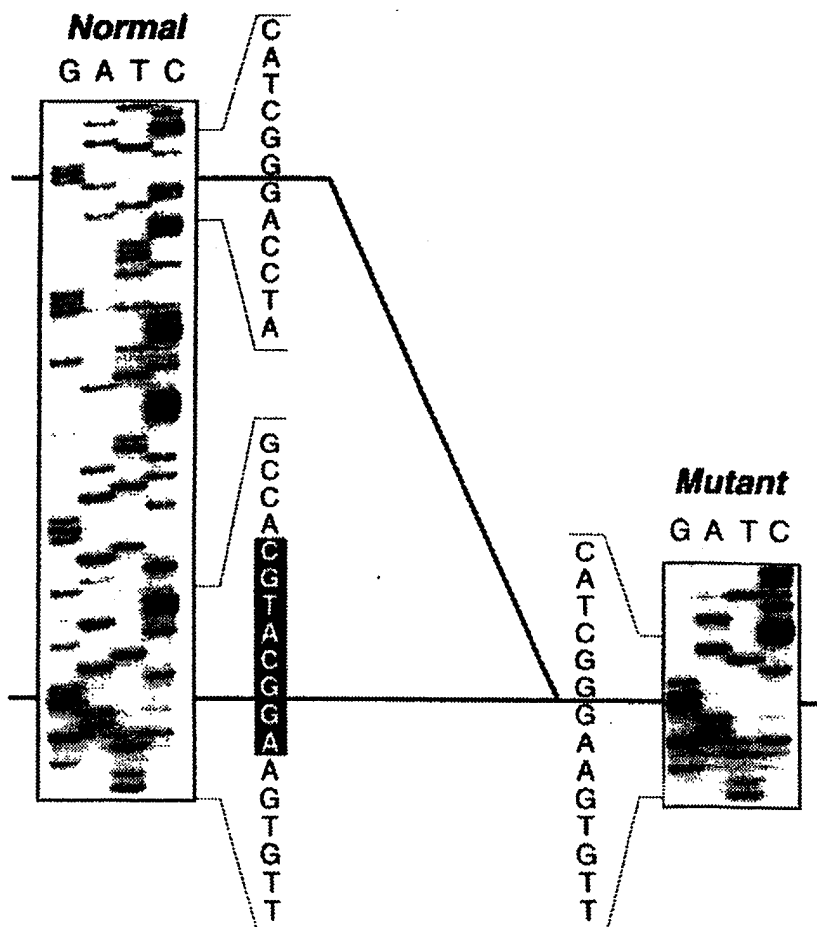
FIG. 7B

MTHFR: KHLKEKVSAGADFIITQLFFEADTFFR
 ||| |· | | |·|||
 DHFR: GHLKLFVT---R-IMQD-FESDTFFP

FIG. 11



Fi . BA



Fi . BB

18/18

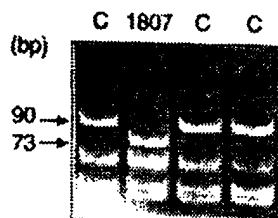


Fig. 9A

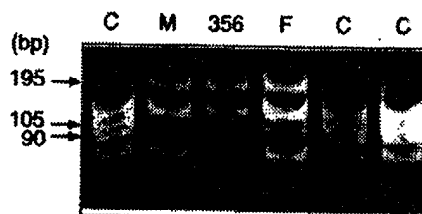


Fig. 9B

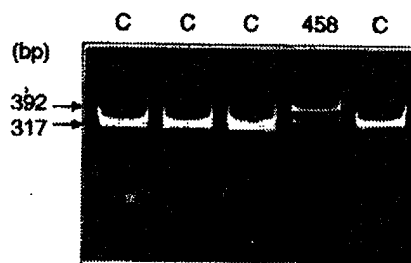


Fig. 9C

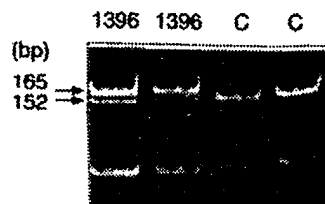


Fig. 9D

INTERNATIONAL SEARCH REPORT

Internati- Application No
PCT/CA 95/00314

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/53 C12N9/06 C12Q1/68 A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|------------|---|-----------------------|
| X | BIOCHEMICAL MEDICINE AND METABOLIC BIOLOGY, vol. 43, no. 3, - June 1990 ACADEMIC PRESS, INC., NEW YORK US, pages 234-242, J. ZHOU ET AL. 'Purification and characterization of methylenetetrahydrofolate reductase from human cadaver liver' the whole document --- -/- | 5 |

☒ Further documents are listed in the continuation of box C.

☐ Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search

3 October 1995

Date of mailing of the international search report

09. 11. 95

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
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Fax: (+ 31-70) 340-3016

Authorized officer

Hornig, H

INTERNATIONAL SEARCH REPORT

Internat Application No
PCT/CA 95/00314

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|------------|---|-----------------------|
| A | METHODS IN ENZYMOLOGY VOLUME 122 VITAMINES AND COENZYMES PART G, - 1986 ACADEMIC PRESS, INC. NEW YORK, US, pages 372-381, R. ROWENA AND G. MATTHEWS 'Methylenetetrahydrofolate reductase from pig liver' the whole document --- | 1-6 |
| A | AM.J. HUMAN. GENET., vol. 48, no. 3, March 1991 AM.SOC.HUM.GENET.,CHICAGO,US, pages 526-545, S.-S. KANG ET AL. 'Thermolabile methylenetetrahydrofolate reductase: An inherited risk factor for coronary artery disease' the whole document --- | 1-6 |
| A | AM. J. OF MEDICAL GENETICS, vol. 45, no. 5, March 1993 WILEY-LISS, INC., NEW YORK, US, pages 572-576, J.C. HAWORTH ET AL. 'Symptomatic and asymptomatic methylenetetrahydrofolate reductase deficiency in two adult brothers' the whole document --- | 1-6 |
| A | NUCLEIC ACIDS RESEARCH, vol. 11, no. 19, 11 October 1983 IRL PRESS LIMITED,OXFORD,ENGLAND, pages 6723-6732, I. SAINT-GIRONS ET AL. 'Nucleotide sequence of metF, the E.coli structural gene for 5-10 methylene tetrahydrofolate reductase and of ist control region' the whole document --- | 1-6 |
| A | MOLECULAR & GENERAL GENETICS, vol. 212, no. 2, May 1988 SPRINGER INTERNATIONAL, AMSTERDAM, NL, pages 246-251, G.V. STAUFFER AND L.T. STAUFFER 'Cloning and nucleotide sequence of the Salmonella typhimurium LT2metF gene and its homology with the corresponding sequence of Escherichia coli' the whole document --- | 1-6 |

INTERNATIONAL SEARCH REPORT

Internati Application No
PCT/CA 95/00314

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|------------|--|-----------------------|
| A | MOL. CELL. BIOL., vol. 4, no. 10, October 1984 ASM WASHINGTON, DC, US, pages 2161-2169, E. YANG AND E.C. FRIEDBERG 'Molecular cloning and nucleotide sequence analysis of the Saccharomyces cerevisiae RAD1 gene' the whole document | 1-10 |
| P,X | --- NAT. GENET. (1994), 7(2), 195-200 CODEN: NGENEC;ISSN: 1061-4036, June 1994 GOYETTE, PHILIPPE ET AL 'Human methylenetetrahydrofolate reductase: isolation of cDNA, mapping and mutation identification' cited in the application the whole document & NATURE GENETICS, vol. 7, no. 4, August 1994 NATURE PUBLISHING CO., NEW YORK, US, page 551 P. GOYETTE ET AL. 'Human mthfr: isolation of cDNA, mapping and mutation identification' see page 551, left column, paragraph 2 | 1-6 |
| T | --- AM.J. HUMAN. GENET., vol. 56, no. 5, May 1995 AM.SOC.HUM.GENET.,CHICAGO,US, pages 1052-1059, P. GOYETTE ET AL. 'Seven novel mutations in the methylenetetrahydrofolate reductase gene and genotype/phenotype correlations in severe methylenetetrahydrofolate reductase deficiency' cited by the applicant the whole document | 1-6 |
| T | --- NAT. GENET. (1995), 10(1), 111-13 CODEN: NGENEC;ISSN: 1061-4036, May 1995 FROSST, P. ET AL 'A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase' cited by the applicant the whole document | 1-6 |

INTERNATIONAL SEARCH REPORT

International application No.

PCT/CA95/00314

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claim 6 is directed to a method of treatment of the human/
animal body, the search has been carried out and based on the alleged
effects of the compound/composition.
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such
an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all
searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment
of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report
covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is
restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.